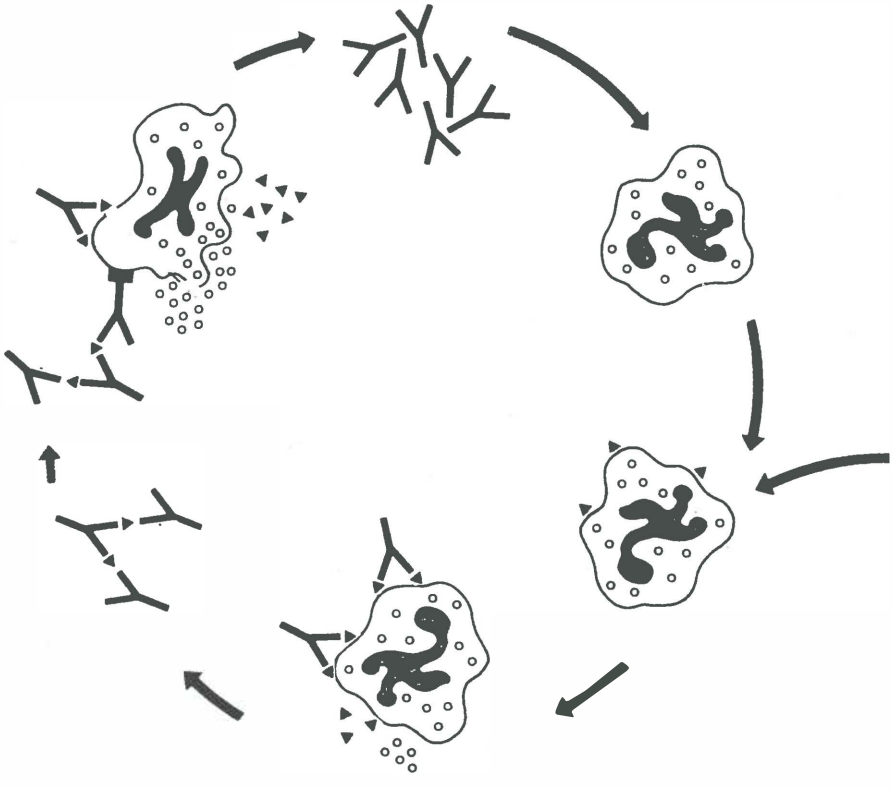
Anti-neutrophil cytoplasmic antibodies in idiopathic inflammatory disorders.



Leontine Mulder

ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES IN IDIOPATHIC INFLAMMATORY DISORDERS.

STELLINGEN

De definitie voor "granulocyt-specifieke antinucleaire antistoffen (GS-ANA) is onjuist omdat zij bij voorbaat al de simultane aanwezigheid van hoogtiterige antinucleaire antistoffen uitsluit.

De meeste granulocyt-specifieke antinucleaire antistoffen zijn anti-neutrofiel cytoplasmatische antistoffen.

ANCA geinduceerde neutrofiel activatie is Fc-afhankelijk; het is een immuuncomplex gemedieerd proces.

De bevolking van Noord-Nederland lijkt meer op de bewoners van Noord-Frankrijk dan op de bewoners van Amsterdam en omstreken, wanneer men de incidentie van anti-neutrofiel cytoplasmatische antistoffen bij patienten met colitis ulcerosa en de ziekte van Crohn in ogenschouw neemt.

Bij de detectie van anti-cathepsine G antistoffen kan men geen gebruik maken van gedecomplementeerde sera.

Statistisch gezien is het bijzonder onwaarschijnlijk dat een vrouw met de naam ANCA een kind met reuma en een kind met een chronische leveraandoening heeft. (Volkskrant 9/4/92)

Het feit dat juist bepaalde autoantigenen in hoge mate vertegenwoordigd zijn onder de eiwitten die homologie vertonen met humaan hsp60 lijkt meer dan slechts een toevalligheid. (D.B. Jones et al., Immunology Today 1993;14:115)

De invloed van het X chromosoom en oestrogeen op zowel de ontwikkeling als het functioneren van de hersenen en in het bijzonder het geheugen pleit voor het bestaan van vrouwelijke intuitie. (J. Collins, Lancet 1993;342:1188)

Indien het oraal toedienen van antigenen zou leiden tot tolerantie-inductie, dan zou voor sommige mensen de melk van de nakomelingen van Herman een uitkomst zijn.

Slordigheid kost veel tijd.

Het dagelijks visualiseren van de juiste slagbeweging leidt tot een aanzienlijke verhoging van je slaggemiddelde.

Stellingen behorend bij het proefschrift "Anti-neutrophil cytoplasmic antibodies in idiopathic inflammatory disorders"
Leontine Mulder
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te Gorinchem

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Een vierjarige periode is (bijna) afgesloten. Het proefschrift ligt er, nu de verdediging nog en *last but not least* het voorwoord. Natuurlijk had ik dit nooit alleen *kunnen* doen, en gelukkig heb ik het ook niet alleen *hoeven* doen. Daarom wil ik iedereen die een bijdrage geleverd heeft aan dit proefschrift bedanken, een aantal van hen in het bijzonder.

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CHAPTER 1

ANTINEUTROPHIL CYTOPLASMIC ANTIBODIES: A STILL-GROWING CLASS OF AUTOANTIBODIES IN INFLAMMATORY DISORDERS.

Cees G. M. Kallenberg, A. H. Leontine Mulder, Jan Willem Cohen Tervaert

Summary

Antineutrophil cytoplasmic antibodies (ANCA) have been described as sensitive and specific markers for active Wegener's granulomatosis (WG). ANCA in WG produce a characteristic staining pattern of neutrophils (c-ANCA) and are directed against proteinase 3 (PR3), a serine proteinase from the azurophilic granules. c-ANCA, more or less equivalent to anti-PR3, occur in more than 90% of patients with extended WG, in 75% of patients with limited WG without renal involvement, and in some 40-50% of patients with vasculitic overlap syndromes suggestive of WG such as microscopic polyarteritis. The presence of c-ANCA is highly specific for those diseases (greater than 98%). Changes of levels of c-ANCA precede disease activity and may be used as guidelines for treatment.
Antibodies producing a perinuclear staining of ethanol-fixed neutrophils (p-ANCA) occur in a wide range of diseases. They are directed against different cytoplasmic constituents of neutrophils. Among those, antibodies to myeloperoxidase are found in patients with idiopathic crescentic glomerulonephritis, the Churg-Strauss syndrome, polyarteritis nodosa with visceral involvement, and vasculitic overlap syndromes. Their specificity for this group of necrotizing vasculitides is high (94 to 99%), although they may occur in patients with hydralazine-induced glomerulonephritis, anti-glomerular basement membrane disease, and possibly in some patients with idiopathic systemic lupus erythematosus. Antibodies to leukocyte elastase are incidentally found in patients with vasculitic disorders, whereas lactoferrin antibodies are detected in patients with primary sclerosing cholangitis with or without ulcerative colitis and in rheumatoid arthritis. Their diagnostic significance awaits further studies. p-ANCA of undefined specificity may distinguish ulcerative colitis (sensitivity of 75%) from Crohn's disease (sensitivity of 20%). p-ANCA also occur in autoimmune liver diseases: in 75% of patients with chronic active hepatitis, in 60% to 85% of those with primary sclerosing cholangitis, and in about 30% of patients with primary biliary cirrhosis. Finally, p-ANCA are detected in chronic arthritides and in some 5% of healthy controls. Assessment of their diagnostic value has to await further characterization of the antigens involved, allowing development of antigen-specific assays.

In 1982, Davies et al (1) reported on the occurrence of autoantibodies to cytoplasmic constituents of granulocytes in the sera of a small group of patients with segmental necrotizing glomerulonephritis. These data went largely unnoticed until 1985, when Van der Woude and colleagues (2) described autoantibodies reacting with the cytoplasm of neutrophils and monocytes as sensitive and specific markers for active Wegener's granulomatosis (WG). The antibodies, when detected by indirect immunofluorescence (IIF) on ethanol-fixed neutrophils, produce a characteristic cytoplasmic fluorescence pattern (cytoplasmic antineutrophil cytoplasmic antibodies or c-ANCA as they are presently designated, figure 1)/ The antigen recognized by c-ANCA proved to be a 29-Kd serine protease from myeloid azurophilic granules, known as proteinase 3 (PR3) (3-5). Testing sera from patients with various forms of vasculitis and glomerulonephritis, it was found that some sera produced a perinuclear to nuclear fluorescence pattern clearly different from the c-ANCA pattern (p-ANCA, figure 1). Initial studies by Falk and Jennette (6) suggested that p-ANCA were directed against myeloperoxidase (MPO), another constituent of azurophilic granules. They detected these antibodies in the sera of patients with idiopathic or vasculitis-associated necrotizing crescentic glomerulonephritis. Recently, p-ANCA were, however, also described in sera from patients with a wide range of different disorders such as ulcerative colitis (7-9), autoimmune liver diseases (10, 11), and rheumatoid arthritis (12-15). These latter p-ANCA do not react with MPO, and their antigenic specificities are largely unknown. Since ANCA were initially described as specific markers for vasculitic disorders, in particular WG, the detection of ANCA in quite different diseases has obscured the value of ANCA testing. The present review attempts to define the current diagnostic significance of ANCA in clinical practice.

C-ANCA and WG.

Sera from patients with active WG display a characteristic staining pattern on ethanol-fixed neutrophils by IIF (2, 16,17). This cytoplasmic or classical ANCA pattern (c-ANCA) is produced by antibodies against PR3, a third serine protease from primary or azurophilic myeloid granules different from elastase and cathepsin G, the two other serine proteases from these granules (3-5). PR3, which has now been cloned (18), is a 29 Kd glycoprotein identical to p 29 b (19), a recently described antibiotic protein from human neutrophils, and myeloblastin (20), a growth-promoting protein from myeloid cells. Thus, c-ANCA recognize a 29 Kd glycoprotein from azurophilic granules with serine protease, antibiotic, and growth-promoting activity.

Until now, most studies dealing with the clinical associations of ANCA have used the IIF technique to discriminate between the WG-associated autoantibodies and other ANCA. Comparison of ANCA detection by IIF with detection by antigen-specific enzyme-linked immunosorbent assays (ELISAs) has indeed shown that the c-ANCA pattern is almost invariably produced by antibodies against PR3 (21,22). Nevertheless, PR3 antibodies may incidentally be detected in sera producing atypical cytoplasmic or perinuclear staining patterns on ethanol-fixed neutrophils. Several ELISA and radioimmunoassay techniques using different antigenic preparations are at present available for the detection of PR3 antibodies (reviewed in (23)). These techniques, however, do not fully compare, and standardization of assays for PR3 antibodies is clearly needed. The availability of cloned PR3 might be helpful in this respect.

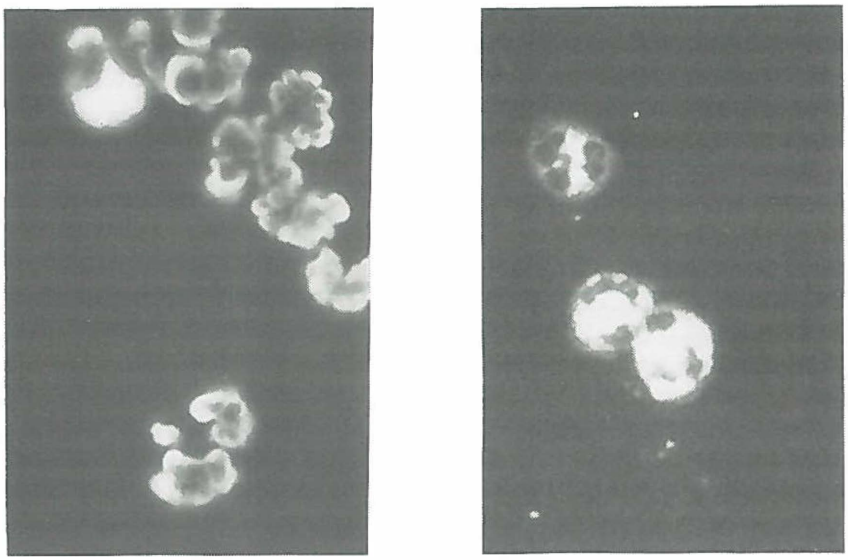

Figure 1. Staining of ethanol fixed neutrophils by indirect immunofluorescence using a serum sample from a patient with active Wegener's granulomatosis that produces a cytoplasmic pattern of fluorescence (c-ANCA, left) and a serum sample from a patient with idiopathic crescentic glomerulonephritis that produces a perinuclear pattern (p-ANCA, right).

c-ANCA or, more specifically, anti-PR3 antibodies are highly sensitive for active WG. In cases of extended WG characterized by the triad of granulomatous inflammation of the respiratory tract, systemic vasculitis, and necrotizing crescentic glomerulonephritis, c-ANCA are present in more than 90% of the patients (2, 16, 17, 24). In patients with so-called limited WG, without renal involvement, c-ANCA are detected in 67% to 86% of the patients (16, 25). When extended WG and limited WG are taken together, the sensitivity of c-ANCA for active disease can be ascertained as 81% (Table 1, and reviewed in (26)). c-ANCA however, have also been described in about 50% of patients with necrotizing (pauci-immune) glomerulonephritis in combination with systemic small-vessel vasculitis but without granulomatous inflammation of the respiratory tract (27). This disease entity, so-called microscopic polyarteritis (MPA) (28), might be included in the spectrum of WG. In fact, some patients who initially present with MPA develop extended WG during a relapse of their disease. In addition, in patients presenting with rapidly progressive glomerulonephritis and/or alveolar haemorrhage, granulomas may be missed in biopsy specimens from the respiratory tract due to incomplete work-up or sampling errors. It should be mentioned that c-ANCA have also been described in 40% to 50% of patients with pauci-immune necrotizing glomerulonephritis without histologic proof of extrarenal granulomatous inflammation or vasculitis (22, 29). Most of these patients, however, have signs of upper airway involvement suggestive of WG (30). In addition, some patients with systemic necrotizing vasculitis involving medium-sized arteries but without histologic proof of WG are positive for c-ANCA. These latter patients also

frequently have signs of upper airway involvement (31) and fulfil the American College of Rheumatology 1990 criteria for the classification of WG (32). Until now, no studies have been published that describe the sensitivity of c-ANCA for active WG as classified according to these recently established criteria. The diagnostic significance of c-ANCA for the disease spectrum mentioned before is determined not only by its sensitivity but also by its specificity for these disorders. In general, sera from selected groups of patients with different forms of vasculitis, renal disorders, and granulomatous diseases were invariably negative for c-ANCA(2,16,17,24, 27,33). When these data are combined, the specificity of c-ANCA for the WG disease spectrum can be ascertained as being more than 98%. In summary, the presence of c-ANCA, more or less equivalent with PR3 antibodies, denotes a spectrum of disease varying from idiopathic pauci-immune necrotizing glomerulonephritis to extended WG.

c-ANCA have also been used to follow disease activity in patients with WG and related disorders. Early reports on ANCA revealed that patients with active disease generally had higher titers of c-ANCA than patients without disease activity, although high titers of ANCA were observed in some patients with inactive disease as well (1,2,16,33,34). A prospective study from our group (17) showed that increases in the ANCA titer preceded relapses of WG by a median of 49 days. Increases in the ANCA titer were not only a sensitive predictor of disease activity but were also highly specific in this regard. These findings were confirmed by others (35). On the basis of these data, a prospective study was undertaken by our group in which patients were randomized for treatment or no treatment once an increase in the ANCA titer had occurred (36). During an observation period of 24 months, ANCA titers rose in 20 of 58 patients. Nine of these patients were assigned to immunosuppressive treatment at the time the ANCA titers had increased, and none of them had relapses. On the contrary, 9 of 11 patients who were not treated at the time their ANCA titers had risen developed a relapse of their disease. Interestingly, the total amount of immunosuppressives used was lower in the former group than in the latter. Thus, serial quantititation of c-ANCA is useful for following and predicting disease activity in WG and may be used as a guideline for treatment.

Table I. Disease associations of anti-proteinase 3 and anti-myeloperoxidase antibodies*

Disease entity	Sensitivity of	
	anti-proteinase 3	anti-myeloperoxidase
Idiopathic crescentic glomerulonephritis	30%	70%
Microscopic polyarteritis	50%	50%
Wegener's Granulomatosis	80%	20%
Churg Strauss Syndrome	10%	70%
Classic polyarteritis nodosa	10%	20%
Polyangiitis overlap syndrome	40%	20%

* These data are summarized from the references cited in the text.

The close association between WG and c-ANCA has led to speculations about the pathophysiologic role of c-ANCA in the disease process (37). Although PR3 antibodies have the capacity to activate primed neutrophils in vitro (38), experimental data to support the pathogenetic role of c-ANCA are at present not available.

p-ANCA in vasculitis, glomerulonephritis and other inflammatory disorders.

During routine IIF testing for ANCA, sera were observed that produced a perinuclear to nuclear fluorescence pattern on ethanol-fixed neutrophils (p-ANCA, figure 1). Of these p-ANCA-positive sera, derived from patients apparently suspected of having vasculitis and/or glomerulonephritis, a considerable number were shown to contain antibodies to MPO (6, 39). MPO, as PR3, is a constituent of the azurophilic granules. How to explain the perinuclear staining pattern of MPO antibodies? This pattern proved to be an artifact of ethanol fixation. MPO, a highly cationic protein, apparently moves and attaches to the negatively charged nuclear membrane during the fixation procedure. When neutrophils are fixed with cross-linking fixatives such as paraformaldehyde, MPO antibodies produce a diffuse granular staining of the cytoplasm (40,41). Because MPO, in contrast to PR3, is commercially available in highly purified form, ELISAs have been developed for the detection of MPO antibodies. Comparison between the immunofluorescence assay and MPO-specific ELISA has shown that (1) only a minority of p-ANCA positive sera derived from groups of patients with a large variety of diseases are positive for MPO antibodies by ELISA (42), and (2) a few sera are positive for MPO antibodies by ELISA but negative for p-ANCA by IIF (43). Thus, in contrast to c-ANCA and PR3 antibodies, the presence of p-ANCA is not at all equivalent with the presence of MPO antibodies.

Clinical association with MPO antibodies.

MPO antibodies are present in nearly all patients with pauci-immune crescentic glomerulonephritis who are negative for c-ANCA/anti-PR3 antibodies (6, 22, 29, 44-46). In one third of the anti-MPO-positive patients, crescentic glomerulonephritis occurs without extrarenal manifestations, so-called idiopathic crescentic glomerulonephritis. Most of the remaining patients have crescentic glomerulonephritis in combination with progressive dyspnea, pulmonary infiltrates, and/or alveolar haemorrhage, or in combination with a systemic illness with constitutional symptoms, arthralgia, purpura, and/or otorhinolaryngologic symptoms (22, 29, 42, 44). Although these symptoms suggest a diagnosis of WG, granulomatous inflammation of the respiratory tract is found only infrequently. A number of these patients have a history of asthma and hypereosinophilia, and fulfil the criteria for the Churg-Strauss syndrome (30-42). MPO antibodies have, however, also been observed in patients with polyarteritis or Churg-Strauss syndrome without renal involvement. In the former group, MPO antibodies were particularly detected in patients with visceral involvement, and not in those with polyarteritis limited to the skin, musculoskeletal system, and peripheral nerves.
At this stage, it should be mentioned that many overlap syndromes occur within the spectrum of vasculitis. Besides well-defined and -delineated disease entities such as extended WG, classic polyarteritis nodosa, and the Churg-Strauss syndrome, overlapping

clinical features of those diseases are seen in individual patients. The term polyangiitis overlap syndrome has been assigned to these diverse clinical presentations (47). A majority of the patients appear to be positive for ANCA. Within this group, PR3 antibodies are associated with clinical features of WG, whereas MPO antibodies are associated with asthma and/or eosinophilia and suggest a diagnosis of the Churg-Strauss syndrome (31). This dissection, however, is far from absolute.

The specificity of MPO antibodies for systemic vasculitis and/or idiopathic crescentic glomerulonephritis has been studied in groups of patients with renal disorders and diseases related to vasculitis and was found as high as 94% to 99% (22, 42, 45). Recently, MPO antibodies were, however, also found in nine patients with hydralazine induced necrotizing and/or crescentic glomerulonephritis (48) and in a substantial number of patients with anti-glomerular basement membrane (GBM) disease (49). Those patients with both anti-GBM antibodies and MPO antibodies frequently have clinical and histologic features that suggest an associated systemic vasculitis (49). Finally, low titers of MPO antibodies have been observed in some sera of patients with systemic lupus erythematosus (SLE) (46, 50, 51). There has been some debate about the possible interaction of DNA/anti-DNA complexes in the ELISA for measuring MPO antibodies. DNA derived from disrupted cells can bind to positively charged MPO. As a result, anti-DNA antibodies may give false-positive results in the ELISA. This item has yet to be studied in more detail.

In summary, MPO antibodies occur in different forms of vasculitis and crescentic glomerulonephritis, and are highly specific for those disorders. Their occurrence in patients with SLE, however, cannot be excluded.

A close correlation between MPO antibodies and disease activity has been found in two studies (42, 46). Levels of MPO antibodies were found higher in sera obtained during active disease than in those obtained during remission (42, 46). In addition, an increase in levels of MPO antibodies prior to disease relapses has been observed (42, 46). At present, however, no prospective studies have been reported in patients with MPO antibodies that demonstrate their value in following and/or predicting disease activity.

p-ANCA produced by antibodies to other lysosomal constituents.

Recently antibodies to leukocyte elastase and lactoferrin have been described that also produce a p-ANCA pattern by IIF on ethanol-fixed neutrophils. Antibodies to elastase, a serine protease from primary granules, occur occasionally in sera from patients with (a presumptive diagnosis of) vasculitis (39, 50, 52) or (drug-induced) systemic autoimmune disease (50, 51), The low prevalence of elastase antibodies limits their diagnostic significance, although they appear to be rather specific for the aforementioned disorders (52). Antibodies to lactoferrin, a constituent of secondary granules, have been mentioned in a few patients with vasculitis (53). Very recently, we observed anti-lactoferrin antibodies in some patients with primary sclerosing cholangitis and/or ulcerative colitis (11, 54) and in a minority of patients with rheumatoid arthritis (55) (see later). Their diagnostic value has not been established as yet.

p-ANCA of undefined specificity

Antibodies that produce a (peri)nuclear pattern on ethanol-fixed neutrophils were, in fact, first described in 1959 by Calabresi et al. (56). These antibodies were detected in patients with rheumatoid arthritis, ulcerative colitis, and chronic hepatitis (56, 57). In contrast to sera of patients with SLE, these latter sera lacked affinity for nucleoproteins of cells other than neutrophils (57). By testing serum samples on different substrates, it was demonstrated that the antibodies in the above-mentioned diseases were reactive with granulocytes only (58-63) or were at least two titersteps higher on granulocytes than on other cells (64). On the basis of these findings, the antibodies were designated as granulocyte-specific antinuclear antibodies (GS-ANA) or antineutrophil nuclear antibodies (see (64) for review). Since the staining pattern produced by GS-ANA positive sera is indistinguishable from the pattern produced by MPO antibodies and elastase antibodies, discussion was conducted during the Second International Workshop on Antineutrophil Cytoplasmic Antibodies (May 1988, Noordwijkerhout, The Netherlands) on whether the term p-ANCA should replace GS-ANA (65). At that time, however, this was believed to be premature, since it was not clear whether GS-ANA as described in the aforementioned diseases recognized nuclear or cytoplasmic antigen(s). Since 1989, several groups have demonstrated that most, if not all, GS-ANA are in fact recognizing cytoplasmic antigens. At first, Saxon et al. (7) showed that the fluorescence pattern on ethanol-fixed granulocytes produced by sera from patients with ulcerative colitis is diffuse cytoplasmic with perinuclear highlighting rather than nuclear. These findings were confirmed by others (8). Using GS-ANA positive sera from patients with ulcerative colitis (66), autoimmune liver diseases (11, 54) and rheumatoid arthritis (12), we could demonstrate that the perinuclear pattern as seen on ethanol-fixed neutrophils turns into a cytoplasmic pattern when neutrophils are fixed with paraformaldehyde. Similar findings were reported by Savige et al. (15) using sera from patients with rheumatoid arthritis. The p-ANCA in the above-mentioned disorders are, in most of the cases, not directed against one of the known lysosomal constituents of myeloid cells. So, further characterization and isolation of the antigen(s) involved are strongly needed. As long as antigen-specific tests are not available in these conditions, p-ANCA have to be detected by the IIF technique. A common problem in the interpretation of results of IIF testing on ethanol-fixed granulocytes is the difficulty in distinguishing p-ANCA from antinuclear antibodies (ANA). p-ANCA can be differentiated from ANA in several ways (13, 67-69). Additional testing of sera that are positive for p-ANCA on ethanol-fixed neutrophils on formalin-fixed cells seems appropriate in this respect ((69), see before).

Clinical significance of p-ANCA of undefined specificity in selected diseases.

The occurrence of p-ANCA (GS-ANA) of undefined antigenic specificity in selected disease groups will be discussed briefly (see also table II).

Patients with colitides/diarrhoeal illnesses: In 1961, Calabresi et al (57) found p-ANCA/GS-ANA in 18 (75%) of 24 sera from patients with ulcerative colitis. These antibodies were not found in sera from 26 patients with other diseases of the colon nor in 300 sera from normal controls (57). These findings were confirmed by others (7-9, 62, 63), although p-ANCA were also detected in 10% to 20% of patients with collagenous

colitis (9) in the latter studies. The combination of a positive result for ANCA testing using a fixed neutrophil ELISA and a positive result for p-ANCA by IIF was found 60% sensitive and 94% specific for ulcerative colitis (9). Thus, ANCA screening may be of value in differentiating ulcerative colitis from other colitides/diarrhoeal illnesses. At present, the relation between ANCA titers and disease activity is not clear. In some studies, a relation between ANCA titers and disease activity has been suggested (8, 57, 63), but this has not been confirmed by others (7, 62).

Patients with autoimmune liver disease: In 1981, Whittingham et al (61) found p-ANCA -/GS-ANA in 36 (72%) of 50 patients with autoimmune chronic active hepatitis but in none of 24 patients with alcoholic hepatitis. The occurrence of p-ANCA in sera from patients with chronic active hepatitis has been confirmed by others (11, 63). p-ANCA were, however, also detected in 60% to 85% of patients with primary sclerosing cholangitis (10, 11, 63) and in 30% to 40% of patients with primary biliary cirrhosis (11, 63), indicating that ANCA screening by IIF is of little value for the differential diagnosis within the spectrum of autoimmune liver diseases. The relation between ANCA and disease activity has only been studies in patients with primary sclerosing cholangitis. A significant correlation was found between ANCA titer and serum levels of aspartate transaminase (63). Recently, we observed that removal of the diseased organ in primary sclerosing cholangitis, i.e. by orthotopic liver transplantation, did not change the ANCA titer (70).

Patients with chronic arthritis: p-ANCA/GS-ANA were first described in 10 patients with rheumatoid arthritis complicated by Felty's syndrome (56). The high prevalence of these antibodies in this condition has been confirmed by others (60). In addition, p-ANCA/GS-ANA can be detected in 50% to 70% of patients with rheumatoid arthritis complicated by vasculitis (13, 71), and in 20% to 40% of patients with uncomplicated rheumatoid arthritis (13, 57, 71). The detection of ANCA is, however, not very helpful in differentiating rheumatoid arthritis from other forms of chronic arthritis, since p-ANCA are also present in 10% to 20% of patients with juvenile chronic arthritis, psoriatic arthritis, and ankylosing spondylitis (72, 73). Titers of p-ANCA/GS-ANA do not appear to parallel rheumatic disease activity (68). The presence of this antibody, however, seems to be related to mor aggressive erosive joint disease (74).

Healthy controls: GS-ANA defined as antibodies reacting with nuclei of myeloid cells only or as ANA whose specific reactivity on myeloid cells surpasses reactivity with nuclei of other cells by at least two dilution steps have been observed in 1% of the serum samples of 20- to 60-year-old normal blood donors, and in 8% of normal controls over the age of 60 (64). Recently, we detected p-ANCA in 12 of 252 sera (5%) of healthy blood-bank donors with a median age of 49 years (range: 18 to 65 years). This, again, underscores the limitations of a positive p-ANCA test in clinical practice.

Conclusions

In summary, until antigen-specific assays for PR3 and MPO antibodies are standardized, ANCA testing by IIF on ethanol-fixed neutrophils is a useful screening method. A positive c-ANCA test result, equivalent in nearly all of the cases with PR3 antibodies, is highly suggestive of a clinical condition within the spectrum of WG. A positive p-ANCA test

result has limited diagnostic significance and should be followed by antigen-specific assays, in particular for MPO. The presence of MPO antibodies is highly suggestive for one of the necrotizing systemic vasculitides including idiopathic necrotizing and crescentic glomerulonephritis. Assessment of the value of ANCA testing for the (differential) diagnosis of other inflammatory diseases of unknown etiology, such as (juvenile) chronic arthritis, inflammatory bowel diseases, and autoimmune liver diseases, has to await further characterization of the antigens involved.

Table II. Occurrence of p-ANCA in selected diseases

Disease	prevalence of p-ANCA	reference
Ulcerative colitis	60-75%	7, 8, 57
Crohn's disease	10-20%	7, 8
Autoimmune chronic active hepatitis	60-70%	11, 61, 63
Primary biliary cirrhosis	30-40%	11, 63
Primary sclerosing cholangitis	60-85%	10, 11, 63
Rheumatoid arthritis		
-complicated by Felty's syndrome	90-100%	56, 60
-complicated by vasculitis	50-75%	13, 71
-uncomplicated	20-40%	13, 57, 71.

CHAPTER 1.2

AIM OF THIS THESIS.

Antibodies that produce a (peri)nuclear fluorescence pattern on ethanol fixed neutrophils were, as stated in the first part of this chapter, first described in 1959 by Calabresi et al. (56). These antibodies were detected in patients with rheumatoid arthritis (and especially those with Felty's syndrome), ulcerative colitis and chronic hepatitis (56, 57). The autoantibodies observed were described as granulocyte-specific antinuclear antibodies (GS-ANA), since they lacked reactivity with nuclei of cells other than granulocytes, or reacted less pronounced with other cells. The target antigen(s) of GS-ANA, however, have never been elucidated (64).

Since the staining pattern produced by GS-ANA positive sera is often indistinguishable from the (peri)nuclear pattern produced by MPO-antibodies and elastase antibodies, it was discussed whether GS-ANA recognize nuclear or cytoplasmic antigen(s). Indeed, since 1989 several groups have demonstrated that most GS-ANA in fact recognize cytoplasmic antigens (7, 8) and thus can be considered as ANCA. However, the antigenic specificity remained unrevealed: it became clear that none of the known lysosomal enzymes recognized by ANCA in systemic vasculitis,i.e. proteinase 3, MPO and elastase, were involved.

This thesis studies the prevalence of ANCA in idiopathic inflammatory disorders in which GS-ANA were described during the 60s: rheumatoid arthritis (***chapter 2***), autoimmune liver diseases (***chapter 3.1***), inflammatory bowel disease (***chapter 4.1 and 4.2***), and juvenile chronic arthritis (***chapter 5***). Different fixation techniques were employed in order to demonstrate that ANCA in those conditions are indeed directed against cytoplasmic constituents of myeloid cells. In all of these studies we related the presence of ANCA to disease activity, disease duration and severity of disease. Interestingly the same pattern was observed for the various diseases: ANCA tend to be present in higher quantity during active disease than in the inactive phase of the disease, and, secondly, the prevalence of ANCA increases with longstanding or extended disease.

ANCA in the chronic inflammatory diseases do not recognize the ANCA antigens known to occur in systemic vasculitis such as proteinase 3, myeloperoxidase and elastase. This thesis describes the partial characterization of the ANCA-antigens involved in these disorders. Lactoferrin can be designated as one of the major antigens recognized, furthermore polypeptides of unknown specificity with molecular weights of 67/66 kD and 63/54 kD can be indicated as ANCA antigens in these inflammatory disorders.

Whether or not ANCA in chronic inflammatory disorders exert a pathogenetic role remains a contentious issue. ***Chapter 3.2*** studies the prevalence of ANCA in patients with primary sclerosing cholangitis (PSC) before and after livertransplantation, to explore whether the autoantibodies are of immunopathogenetic importance and to study the influence of the diseased organ on the regulation of the autoantibody synthesis. ***Chapter 6.1*** evaluates whether ANCA in sera from patients with inflammatory disorders can initiate the respiratory burst in primed neutrophils, an event that may be relevant for the persistence of the inflammatory process. The final study of this thesis (***chapter 6.2***) explores whether or not specific subclasses of ANCA are involved in neutrophil activation.

So, in summary this thesis evaluates the hypothesis that ANCA in chronic inflammatory disorders are, on the one hand, a corollary of chronic inflammation, but on the other hand, contribute to the chronicity of the disease. In particular, the following items are adressed:

1. How do the antigenic specificities of ANCA compare in chronic inflammatory conditions such as rheumatoid arthritis, autoimmune liver diseases, inflammatory bowel disease and juvenile chronic arthritis?
2. What is the relation between the presence and characteristics of ANCA and disease pattern, activity, and duration in these disorders?
3. What is the possible pathogenetic significance of ANCA in those disorders, i.e. how do ANCA contribute to the inflammatory process?

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CHAPTER 2

ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES IN RHEUMATOID ARTHRITIS: CHARACTERIZATION AND CLINICAL CORRELATIONS

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Summary

Objective: To study the prevalence, interrelationships, and target antigens of anti-neutrophil cytoplasmic antibodies (ANCA) in rheumatoid arthritis (RA) and to relate their presence to disease duration and occurrence of extra-articular manifestations, including vasculitis.

Methods: Sera from 94 patients with RA (31 with recent onset disease, 35 with longstanding disease but without extra-articular manifestations, and 28 with extra-articular disease) were studied for the presence of ANCA by indirect immunofluorescence. All sera were tested by Elisa for the presence of antibodies to proteinase 3, myeloperoxidase (MPO), elastase, lactoferrin (LF), and cathepsin G (CG), and by Western blotting for antibodies to neutrophil proteins.

Results: Seventy percent of the 94 sera showed staining of the nuclei of ethanol fixed neutrophils; 32% of the 94 were proven to have ANCA, as manifested by their cytoplasmic staining pattern on paraformaldehyde fixed neutrophils. In the Elisa, 19 sera reacted with LF, 1 with MPO, and 1 with CG. By Western blotting, 21 sera reacted with LF, and 15 reacted with previously unknown polypeptides (7 sera with a 67/66 kD doublet and 8 sera with a 63/ 54 Kd doublet). Neither of these antibodies was associated with a particular subset of the disease, but the prevalence of the antibodies tended to increase among patients with longstanding disease.

Conclusion: ANCA in RA are directed toward diverse cytoplasmic antigens of the neutrophil, in particular, LF and other, not yet fully characterized polypeptides. The antibodies are not a marker for a disease subset, but are probably a corollary of chronic inflammation.

Antibodies to neutrophil cytoplasmic components (ANCA) have been extensively described as sensitive and specific markers for systemic vasculitis and (idiopathic) crescentic glomerulonephritis (for review, see refs. 1 and 2). By indirect immuno-fluorescence (IIF) on ethanol fixed neutrophils, at least two types of ANCA can be distinguished: one showing a characteristic cytoplasmic fluorescence pattern (c-ANCA) (in most cases produced by antibodies to proteinase 3 (anti-PR3))(3) and strongly associated with Wegener's granulomatosis (4-8), and another showing a perinuclear to nuclear pattern (p-ANCA) (in many cases produced by antibodies to myeloperoxidase (MPO)) (5, 8, 9). The presence of anti-MPO antibodies (5, 7-9), but not the presence of p-ANCA alone (10), is associated with crescentic glomerulonephritis and other forms of vasculitis.
Recently, p-ANCA have been detected in sera from patients with ulcerative colitis, to a lesser extent those with Crohn's disease, in patients with autoimmune liver diseases such as primary sclerosing cholangitis and autoimmune chronic active hepatitis, and in a number of diverse conditions such as Kawasaki's disease, Felty's syndrome, Sweet's syndrome and acquired immunodeficiency disease (11-20). In rheumatoid arthritis (RA) the presence of granulocyte-specific antinuclear antibodies (GS-ANA) was observed long before ANCA were recognized. As early as 1966, Faber and Elling (21) described GS-ANA in sera from patients with RA. Further studies confirmed that GS-ANA occur frequently in sera and synovial fluids from adults with RA (22-27). GS-ANA are defined as antinuclear antibodies whose reactivity with myeloid cells surpasses reactivity with other cells by at least 2 dilutions (27).
The target antigen(s) of GS-ANA have not been elucidated (27). However, GS-ANA are indistinguishable from p-ANCA by IIF on ethanol fixed granulocytes, suggesting that the 2 antibodies recognize identical antigens which, by analogy to MPO, may be cytoplasmic in nature. Indeed, Savige et al. (28) reported the presence of ANCA in 12% and GS-ANA in 14% of their RA patients, comparable with the 13% prevalence reported by Lassoued et al. (29). Both ANCA and GS-ANA were associated with vasculitis. A weak association between GS-ANA and vasculitis in RA was also found by Nässberger et al. (30), although other authors (31) have reported that ANCA positivity occurs with equal frequency among RA patients with and those without vasculitis.
In view of these conflicting data, we undertook the present study to elucidate the localization and the nature of the antigen(s) recognized by ANCA/GS-ANA in RA. In addition, we sought correlations between the presence of the antibodies and the occurrence of extra-articular manifestations and the duration of disease, in order to evaluate their early diagnostic and prognostic significance. The results showed that GS-ANA can indeed be considered as ANCA, recognizing cytoplasmic antigens, in particular lactoferrin (LF) and 2 sets of polypeptides not previously described. The occurrence of the autoantibodies in RA was not associated with extra-articular manifestations, and their incidence tended to increase with increasing duration of disease.

Material and methods

Patients and sera:

Sera from 94 consecutive patients with RA were studied. All patients met the American

College of Rheumatology (formerly, the American Rheumatism Association) criteria for the diagnosis of RA (32). The group of patients was divided into 3 subpopulations: 1) patients with recently diagnosed RA (n=31), i.e., < 2 years (median 0.5 years); 2) patients with extra articular manifestations (n=28), i.e., vasculitis (n=9), subcutaneous nodules (n=12), pericarditis (n=4), pleuritis (n=3), or episcleritis (n=2), alone or in combination; and 3) patients without extra articular manifestations and with a disease duration of > 2 years (n=35). Characteristics of the patients are shown in table 1. Synovial fluid samples from 6 patients, obtained during active disease, were studied as well.

Control sera were obtained from 252 healthy bloodbank donors (137 men, 115 women; median age 49 years (range 18-65 years)) and from patients with ANCA of defined specificity (anti-MPO (n=3), anti-PR3 (n=3), anti-human leucocyte elastase (anti-HLE) (n=1), and anti-LF (n=1)). In addition, reference sera from patients with ANA of defined specificities were included (anti-double stranded DNA (anti-dsDNA), anti-extractable nuclear antigen (anti-ENA) (Sm, U_1-RNP, SS-B), and anti-histone).

Mouse monoclonal antibodies (MAbs) were used directed against myeloperoxidase (14.15, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands), proteinase 3 (12.8, CLB, Amsterdam, The Netherlands) and elastase (M752, Dakopatts, Copenhagen), and polyclonal antibodies directed against lactoferrin (A186, Dakopatts, Copenhagen, Denmark) or cathepsin G (219358, Calbiochem, La Jolla, CA).

Table I. Demographic and clinical characteristics of 94 patients with RA grouped according to disease duration and presence of extra-articular manifestations

	RA of recent onset	RA with extra-articular manifestations[4]	Longstanding RA without extra-articular manifestations
	n=31	n=28	n=35
male/female	12/19	13/15	10/25
age[1]	44 (19-69)	59 (30-75)	55 (15-78)
disease duration[1]	0.5 (0-2)	12 (0-27)	13 (3-36)
immunosuppressive drugs[2]	2	9	10
IgM rheumatoid factor[3]	205 (5-1300)	1093 (1-5000)	262 (0-1800)

[1] given in years, mean (range)
[2] given is the number of patients either using prednisolone, azathioprine, or methotrexate
[3] mean titer in IU/ml (range)
[4] this population includes RA patients with either vasculitis (n=9), subcutaneous nodules (n=12), pericarditis (n=4), pleuritis (n=3) or episcleritis (n=2) alone or in combination.

Indirect Immunofluorescence:

Testing for ANCA was done according to Wiik (33) as agreed on the first international workshop on ANCA with minor modifications (34). 1:16 to 1:512 serial dilutions in PBS

of patient or control sera were tested. Slides were read by two independent observers not aware of the clinical diagnosis. A titer of $\geq$ 1:32 was considered positive. Fluorescence patterns were classified as classical or c-ANCA, perinuclear or p-ANCA, and atypical, i.e. positive fluorescence different from c-ANCA or p-ANCA (33). Testing for antinuclear antibodies was performed by indirect immunofluorescence on human fibroblasts, as described before (35).
To study whether the antigens recognized by p-ANCA were artificially redistributed during ethanol fixation, phosphate buffered paraformaldehyde (0.5%) at pH 8.5 (10', RT) was additionally used to fix the granulocytes, after which detection of ANCA was performed as described above. In order to evaluate the influence of paraformaldehyde fixation on the localization of nuclear antigens under the conditions described above, we also applied the latter fixative for ANA testing.

Elisa:
An antigen capture Elisa (8) was used to test sera for the presence of either anti-proteinase 3 (anti-PR3), anti-myeloperoxidase (anti-MPO) or anti-elastase (anti-HLE) antibodies. The presence of anti-lactoferrin or anti-cathepsin G antibodies was detected by Elisa directly coated with lactoferrin (LF) (Serva, Heidelberg, Germany), at a concentration of 7 μg/ml in PBS, or with cathepsin G (CG) (Calbiochem, La Jolla, CA), at a concentration of 1 μg/ml in 0.1 M bicarbonate buffer (pH 9.6). Anti-tetanus antibodies were measured according to routine procedures as described previously (36). Results were considered positive when the value obtained exceeded the mean of 25 normal control sera by more than 3 SD.

Western Blotting:
SDS-PAGE was performed using the method of Laemmli. The neutrophil suspension (2.10^8 cells/ml) was sonificated 3 x 20'' in 1M NaCl containing 5 mM phenylmethylsulfonylfluoride (PMSF, Sigma Chemical Co., St. Louis, MO). Membrane fragments were removed by ultracentrifugation and an extract equivalent to 6.10^7 cells was applied on 10% polyacrylamide gels under denaturing but non-reducing conditions. Subsequently Western blotting was performed according to standard procedures. Serum samples were applied at a dilution of 1:50, synovial fluid samples were treated with hyaluronidase (10 U/ml, 1 hr, 37 °C) before application and used in the same dilution as the serum samples. ANCA of defined specificity (MPO, PR3), mono- or polyclonal antibodies directed against MPO, PR3, HLE, LF or CG, and serum samples of healthy blood bank donors were used as controls.
To control for myeloid specificity, a protein extract of HEp2 cells was made following exactly the same procedures. Western blots of the extract from HEp2 cells were used as negative controls.

Statistics:
Statistical analysis was performed using the χ square test.

Results

Detection of ANCA by indirect immunofluorescence:
Sera of 94 consecutive RA patients were screened for the presence of ANCA by IIF on ethanol fixed granulocytes. 66 RA sera (70%) produced fluorescence on ethanol fixed granulocytes, the fluorescence pattern invariably being (peri)nuclear.
To study the possibly cytoplasmic nature of the antigen(s) involved, we tested all 94 RA sera on paraformaldehyde fixed neutrophils. Thirty sera (32%) produced a cytoplasmic fluorescence pattern (table 2) of which 5 sera had been scored negative on ethanol fixed cells. The cytoplasmic fluorescence pattern on paraformaldehyde fixed neutrophils was indistinguishable from that produced by antibodies to myeloperoxidase and elastase, which also produced a (peri)nuclear pattern on ethanol- and a cytoplasmic pattern on paraformaldehyde fixed neutrophils. ANA reference sera of defined specificity (i.e. anti-ds DNA, anti ENA (Sm, U_1-RNP, and SS-B), and anti-histone) did not produce cytoplasmic staining on paraformaldehyde fixed neutrophils, but did show their characteristic nuclear fluorescence pattern on fibroblasts, regardless of the fixative used. Thirty sera from healthy controls were negative on paraformaldehyde fixed granulocytes.

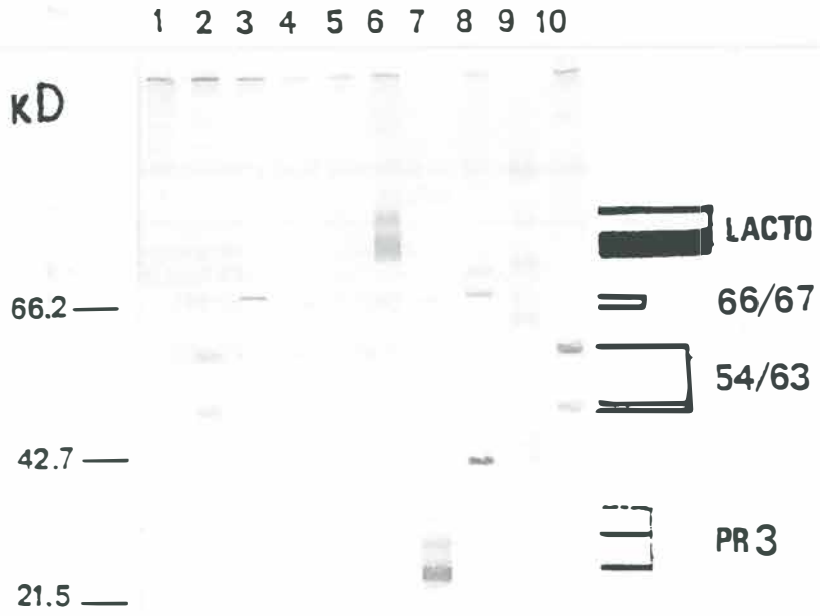

Figure 1. Western blot using sonicated neutrophils as antigenic substrate. Lane 1: buffer control; lane 2 and 10: sera with α-63/54 kD antibodies; lane 3 and 8: sera with α-67/66 kD antibodies; lane 4: serum with α-63 kD antibodies; lane 5: negative control serum; lane 6: serum with α-lactoferrin antibodies; lane 7: monoclonal anti-proteinase 3 antibody; lane 9: serum with myeloperoxidase antibodies.

Detection by Elisa and Western Blotting:
All sera were analyzed by Elisa for antibodies to defined neutrophil cytoplasmic antigens,

i.e. proteinase 3 (PR3), myeloperoxidase (MPO), elastase (HLE), lactoferrin (LF), and cathepsin G (CG). Antibodies to lactoferrin were found in nineteen sera, and anti-myeloperoxidase and anti-cathepsin G antibodies in one serum each.
By Western Blotting using sonified neutrophils as antigenic source mono- and/or polyclonal antibodies to myeloperoxidase, proteinase 3, elastase, lactoferrin and cathepsin G all showed reactivity with proteins of the expected molecular weight (fig 1). Lactoferrin was recognized in Western Blot by 21 sera (23%) of which 17 showed a perinuclear fluorescence pattern on ethanol fixed granulocytes (table 3). All sera positive for anti-lactoferrin by Elisa were positive on Western Blot as well. The two sera reacting by Elisa with MPO and CG, respectively, were also found to react with MPO and CG in Western Blot. Furthermore, Western Blotting revealed reactivity of sera with a protein doublet of 67 and 66 kD and with a fixed combination of 63 and 54 kD polypeptides (fig 1). Seven sera reacted with the protein doublet of 67 and 66 kD, five of them producing a perinuclear fluorescence on ethanol fixed granulocytes. Eight sera reacted with the two proteins of 63 kD and 54 kD. Six of these latter 8 sera showed a perinuclear fluorescence on ethanol fixed granulocytes. Sera of normal healthy bloodbank donors (n=60) did not show reactivity with any of the aforementioned proteins by Western Blotting.
In order to study the specificity for myeloid cells of these proteins recognized by RA sera, we tested the sera also for reactivity with sonificated HEp2 cells in Western blot. The sera did not display reactivity with any protein in the range of lactoferrin or the other aforementioned proteins (results not shown).

Table II. Prevalence of ANCA as detected by IIF on differently fixed granulocytes in subpopulations of RA patients.

	p-ANCA[1]	cytoplasmic staining on PF-fixed granulocytes[2]
	n= (%)	n= (%)
total RA population n=94	66 (70%)	30 (32%)
RA of recent onset n=31	20 (65%)	7 (23%)
RA with extra-articular manifestations n=28	18 (64%)	5 (18%)
Longstanding RA without extra-articular manifestations n=35	28 (80%)	18 (51%)
Healthy controls n=250	12 (5%)	0 (0%)[3]

[1] number of sera showing perinuclear fluorescence on ethanol fixed granulocytes
[2] number of sera showing cytoplasmic fluorescence on paraformaldehyde (PF) fixed granulocytes
[3] thirty samples tested were found negative

Table III. Specificity of ANCA as determined by Western blotting in RA patients grouped according to disease duration and presence of extra-articular manifestations.

	specificity of ANCA by Western blotting					
	α-lactoferrin		α-67/66 kD		α-63/54 kD	
	p-ANCA +	p-ANCA -	p-ANCA +	p-ANCA -	p-ANCA +	p-ANCA -
total population (n=94)	17	4	5	2	6	2
RA of recent onset (n=31)	3	1	1	0	1	1
RA with extra-articular manifestations (n=28)	5	2	2	1	1	1
Longstanding RA without extra-articular manifestations (n=35)	9	1	2	1	4	0

Clinical associations:

The prevalence of ANCA, whether p-ANCA on ethanol- or cytoplasmic ANCA on paraformaldehyde fixed neutrophils, was not increased in patients with extra-articular manifestations compared to those without (table 2). The same applied to the subgroup of patients with vasculitis. Also, antibodies to defined antigens as tested by Elisa and Western blotting, in particular antibodies to lactoferrin, were not increased in patients with extra-articular disease, nor in the subgroup with vasculitis (table 3). Titers of ANCA and levels of anti-lactoferrin antibodies did not differ between the groups of patients with early onset, longstanding, and extra-articular disease, respectively. In each group median titer was 64 (range 32-$\geq$512). Patients with longstanding disease, however, tended to have a higher prevalence of ANCA compared to patients with early disease. This difference was significant ($p<0.05$) for ANCA as detected on paraformaldehyde fixed granulocytes.

The presence of ANCA was not related to the use of immunosuppressive drugs nor to the level of C-reactive protein as studied in the group with RA of recent onset.

Detection of anti-lactoferrin antibodies in the synovial fluid:

In order to evaluate whether ANCA in RA were present not only in plasma samples of RA patients but at the site of inflammation as well, six synovial fluid samples from patients with anti-lactoferrin antibodies were tested both by Elisa and Western blotting for the presence of these antibodies. In all cases anti-lactoferrin antibodies were present both in serum and synovial fluid. Since the synovium can be a site for antibody production, anti-lactoferrin antibody levels were compared to anti-tetanus antibody levels in synovial fluid and serum in order to reveal whether the anti-lactoferrin antibodies were locally produced in the synovium. Anti-tetanus antibodies were chosen as reference antibodies since local production of antibodies against a homogenous recall antigen would not be

expected to occur preferentially at the synovial site. Roughly the same ratios of anti-lactoferrin and anti-tetanus antibodies were found in serum samples and synovial fluids.

Discussion

The present study demonstrates that 70% of 94 consecutive RA sera stained the nuclei of ethanol fixed granulocytes by IIF of which 36% were directed to cytoplasmic constituents of the granulocyte, as manifested by their cytoplasmic staining pattern on paraformaldehyde fixed neutrophils. The autoantigens involved are, in particular, lactoferrin and two sets of polypeptides not previously described. The presence of these autoantibodies was not associated with extra-articular manifestations, but their prevalence tended to increase with longstanding disease.

As has been shown by Charles et al. (37) anti-MPO antibodies stain the nuclei of ethanol fixed granulocytes due to artificial redistribution of the antigen during ethanol fixation. Probably, ionic interactions between the negatively charged nucleus and the cationic protein MPO leads to binding of MPO to the nucleus in improperly fixed cells. Since crosslinking fixatives do not allow shifting of antigens towards the nuclear membrane during fixation, we used paraformaldehyde fixed granulocytes to study the presumably cytoplasmic nature of the antigen(s) recognized by the antibodies in the 94 RA sera. Thirty sera (32%) displayed diffuse granular cytoplasmic fluorescence indistinguishable from that produced by antibodies to MPO on the latter substrate. Strikingly, not all of those 30 sera reacted with ethanol fixed neutrophils. Probably, the antigen(s) are readily extracted from the cell and might thus be lost during ethanol fixation in accordance with the observations by Wiik (27) during his studies on GS-ANA. We, also, have observed that some sera positive for anti-MPO in high titer by Elisa are negative on ethanol fixed granulocytes but positive on paraformaldehyde fixed cells (38). Crosslinking fixatives as paraformaldehyde probably induce a stronger fixation of certain antigens. The nuclear pattern produced by sera negative on paraformaldehyde fixed cells is, probably, related to the presence of ANA in a considerable number of those sera, although discrepancies were observed. Thus, GS-ANA described in previous studies (21-27) generally can be considered as ANCA.

To elucidate the nature of the cytoplasmic antigen(s) recognized by ANCA in RA characterization studies were performed. Antigen-specific Elisa's showed that ANCA in rheumatoid arthritis do not recognize either proteinase 3 or elastase, antigens that are recognized by ANCA in Wegener's Granulomatosis (3, 39). Only one serum contained antibodies to MPO, which are associated with idiopathic crescentic glomerulonephritis and different forms of necrotizing systemic vasculitis (8, 9). One serum was positive for antibodies to cathepsin G, and 19 sera contained antibodies to lactoferrin. Most of the sera positive by Elisa were positive also for ANCA by IIF on paraformaldehyde fixed cells showing a cytoplasmic fluorescence pattern. To further characterize the antigenic specificity of the remaining ANCA positive sera Western blot analysis was performed on all sera.

Apart from ANCA recognizing lactoferrin, we could identify two other distinct

specificities of ANCA: one group of sera recognized two polypeptides of 67 and 66 kD, and another group recognized two polypeptides of 63 and 54 kD. These latter two specificities of ANCA have not been described before and seem to be specific for myeloid cells since the antigens were not present in HEp2 cell extracts. In addition, the 67/66 kD polypeptide probably is a granular protein, since it can be demonstrated to be present in a degranulate of PMNs (data not shown).
Correlation between Elisa and Western blot techniques proved to be good: sera positive for MPO and cathepsin G by Elisa showed reactivity with proteins of the expected size by blotting, and all sera positive for anti-lactoferrin by Elisa reacted with lactoferrin on Western blot. Two more sera reacted with lactoferrin on Western blot, demonstrating the higher sensitivity of the blot technique compared to Elisa.
Interestingly, reactivity with lactoferrin, the 67/66 kD doublet and the 63/54 kD polypeptide combination has also been observed analyzing ANCA occurring in juvenile chronic arthritis (manuscript in preparation), inflammatory bowel disease (40), and autoimmune liver disease (16). Those ANCA might thus be a marker of idiopathic chronic inflammation in an autoimmune environment. This hypothesis is supported by the observation that ANCA in RA tended to be associated with more longstanding disease, in accordance with the finding in autoimmune liver diseases in which the presence of ANCA was associated with cirrhosis as a marker of longstanding disease (16). In contrast to many other autoantibodies, ANCA in RA are thus not a marker or predictor of early disease. Whereas earlier reports (30, 31) have detected an association of ANCA, in particular anti-lactoferrin antibodies, in RA with vasculitis, we did not find an association with vasculitis nor with extra-articular disease. Patient selection or differences in methodology might underlie these discrepancies.

The pathogenetic role of ANCA is still unclear. Possibly, these autoantibodies are just an epiphenomenon of chronic inflammation. Falk et al. (41), however, showed that ANCA recognizing proteinase 3 and MPO can induce the respiratory burst and degranulation of primed neutrophils in vitro. Initial studies in our laboratory have shown that anti-lactoferrin antibodies from RA patients are also capable to induce activation of primed granulocytes. Since anti-lactoferrin antibodies are present in synovial fluid samples as shown in this study, a vicious circle is suggested: at the inflammatory site the presence of the antibodies leads to enhanced release of auto-antigens in combination with the production of reactive oxygen radicals. Since lactoferrin is known to be present in the synovium at very high levels compared to its levels in plasma, we hypothesized that the synovium might be a place of preferential production of autoantibodies to lactoferrin. Ratios of anti-lactoferrin and anti-tetanus antibodies, however, were approximately the same in serum and synovial fluid samples, making local production of anti-lactoferrin antibodies in the synovium less probable. Anti-lactoferrin antibodies may, however, participate in immune complex formation in the synovium, which may lead to underestimation of its levels in the synovial fluid and which may contribute to the inflammatory process. Although the presence of ANCA nor their titer seemed to be associated with disease activity, longitudinal studies are required to determine whether an increase in titer precedes a relapse of the disease, as was noticed by some authors (7, 42), but not by others (43) in Wegener's Granulomatosis with respect to anti-proteinase 3 antibodies.

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CHAPTER 3.1

PREVALENCE AND CHARACTERIZATION OF NEUTROPHIL CYTOPLASMIC ANTIBODIES IN AUTOIMMUNE LIVER DISEASES.

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Summary

To evaluate the diagnostic significance of neutrophil cytoplasmic antibodies in chronic liver diseases we assessed the prevalence of neutrophil cytoplasmic antibodies in autoimmune liver diseases, in particular in primary sclerosing cholangitis, autoimmune chronic active hepatitis and primary biliary cirrhosis, and we also determined the specificity of perinuclear-pattern neutrophil cytoplasmic antibodies for those autoimmune liver diseases by testing sera from patients with non-autoimmune chronic liver diseases. Neutrophil cytoplasmic antibodies were detected in 79% of sera from patients with primary sclerosing cholangitis (n=24), in 88% of sera from patients with autoimmune chronic active hepatitis (n=24), and in 28% of sera from patients with primary biliary cirrhosis (n=25). The presence of neutrophil cytoplasmic antibodies in these diseases correlated significantly ($p < 0.008$) with the presence of cirrhosis. Neutrophil cytoplasmic antibodies were not detected in non-autoimmune liver diseases. All neutrophil cytoplasmic antibody-positive sera produced a perinuclear fluorescence pattern on ethanol fixed granulocytes. On neutrophils fixed with paraformaldehyde, a granular cytoplasmic immunofluorescence pattern was observed, demonstrating the cytoplasmic nature of the antigen or antigens involved.

Further characterization studies showed that neutrophil cytoplasmic antibodies in autoimmune liver diseases are not directed against myeloperoxidase, proteinase 3, or elastase, the neutrophil cytoplasmic antibody specificities associated with necrotizing vasculitis, glomerulonephritis or both. On Western blots neutrophil cytoplasmic antibodies in autoimmune liver diseases showed reactivity with either lactoferrin, a 67/66 Kd protein combination, or a 40 Kd polypeptide. Reactivity with either of these proteins was observed in sera from patients with primary sclerosing cholangitis (38%), autoimmune chronic active hepatitis (17%), and primary biliary cirrhosis (20%). Because the same specificities have been found in neutrophil cytoplasmic antibody positive sera from patients with inflammatory bowel disease and rheumatoid arthritis, these data suggest that these neutrophil cytoplasmic antibodies are a corollary of chronic idiopathic inflammation.

Antibodies to neutrophil cytoplasmic components (ANCA) have been extensively described as sensitive and specific markers for systemic vasculitis and (idiopathic) crescentic glomerulonephritis (1, 2). By indirect immunofluorescence (IIF) on ethanol fixed neutrophils, two staining patterns of ANCA can be distinguished: a characteristic cytoplasmic pattern (c-ANCA) produced by antibodies to proteinase 3, which are strongly associated with Wegener's granulomatosis, and a perinuclear to nuclear pattern (p-ANCA), in many cases produced by antibodies to myeloperoxidase. The latter antibodies are associated with idiopathic crescentic glomerulonephritis and different forms of necrotizing systemic vasculitis (3-5). The p-ANCA pattern produced by myeloperoxidase antibodies has been shown to be an artefact of ethanol fixation. After fixation of neutrophils with cross-linking fixatives such as paraformaldehyde, the antibodies produce a granular staining of the cytoplasm (6).

Recently, p-ANCA have been detected in sera of patients with ulcerative colitis (UC) and, to a lesser extent in Crohn's disease (CD), but not in other diarrheal diseases (7-11). As a link to inflammatory bowel disease (IBD), p-ANCA have also been described in primary sclerosing cholangitis (PSC). Duerr et al. (9) reported 33 out of 51 PSC patients (65%) to be p-ANCA positive, and Klein et al.(12) found 87% of their PSC patients to be positive for p-ANCA. Also, p-ANCA have been found in other autoimmune liver diseases such as autoimmune CAH (AI-CAH) and PBC, but controversy exists about the prevalence of ANCA in these diseases (9,12,13). Different methods of detection may in part explain the observed discrepancies.

The nature of the antigen or antigens recognized by p-ANCA in IBD and autoimmune liver diseases has not been elucidated so far, although it has been reported that these p-ANCA do not recognize myeloperoxidase, elastase or cathepsin G (10).

To evaluate the diagnostic significance of p-ANCA within the group of chronic liver diseases, we assessed the prevalence of p-ANCA in autoimmune liver diseases; in particular, we assessed the prevalence in PSC, AI-CAH and PBC and determined the specificity of p-ANCA for the group of autoimmune liver diseases by also testing sera from patients with non-autoimmune chronic liver diseases, such as viral hepatitis, alcoholic cirrhosis, Wilson's disease and α_1-antitrypsin deficiency. To ascertain the cytoplasmic nature of the antigens involved, we detected ANCA by IIF both on ethanol fixed granulocytes and on paraformaldehyde fixed granulocytes. In addition, we studied the antigenic specificities of p-ANCA in these disorders by Elisa using well-defined myeloid proteins and by Western blotting techniques using a neutrophil extract as antigenic source.

Patients and methods

Patients and sera:

Sera were studied from patients with PSC (n=24), patients with AI-CAH (n=24) and patients with PBC (n=25). Disease controls consisted of patients with chronic viral hepatitis (n=7), alcoholic cirrhosis (n=6) and patients with metabolic and other chronic liver disorders, including Wilson's disease (n=2), α_1-antitrypsin deficiency (n=2), erythropoietic protoporphyria (n=2), Budd-Chiari syndrome (n=3), and hemangioma

Table I. Characteristics of the patients grouped according to disease.

Diagnosis[1]	PSC	AI-CAH	PBC	Viral	Alcoholic	Metabolic and other
Number of patients	24	24	25	7	6	10
Sex (M/F)	16/8	8/16	2/23	6/1	4/2	3/7
median age (year) (range)	36 (22-57)	33 (17-69)	52 (41-73)	56 (40-60)	48 (40-60)	33 (19-60)
presence of cirrhosis n, (%)	20 (83)	19 (79)	15 (60)	6 (86)	6 (100)	9 (90)
conconmitant presence of UC n, (%)	11 (46)	3 (13)	0	0	0	0
cumulative presence of [3]						
ANA pos n, (%)	5 (21)	10 (63)	12 (48)	0	0	0
AMA pos n, (%)	0	0	24 (96)	0	0	0
no. of patients of immuno-suppression[4] n, (%)	2 (8)	18 (75)	1 (4)	0	0	0
prednisolone dose (mg)	10	10 (0-15)	10			
azathioprine dose (mg)	0	50 (0-100)	0			

[1] PSC: primary sclerosing cholangitis, AI-CAH: autoimmune chronic active hepatitis, PBC: primary biliary cirrhosis, viral: viral hepatitis, alcoholic: alcoholic cirrhosis
[2] Budd Chiari syndrome (n=3), Wilson's Disease (n=2), erythropoietic protoporphyria (EPP, n=2), α1-AT deficiency (n=2), and haemangioma (n=1).
[3] ANA = antinuclear antibodies, AMA = antimitochondrial antibodies
[4] median dose (range)

(n= 1). Diagnoses were based on well accepted clinical, serological, histopathological and radiological criteria. In particular, diagnosis for AI-CAH was established by the combination of histological findings; duration of disease of at least 6 months; absence of other causes of chronic liver disease such as metabolic, drug-related and viral causes including hepatitis B and hepatitis C virus; presence of hypergammaglobulinemia; and transaminase levels elevated to at least five times normal (14). Characteristics of the patients are given in table 1. The disease was in the stage of cirrhosis in 83% of PSC patients, 79% of AI-CAH patients, 60 % of PBC patients, and in 95% of non-autoimmune chronic liver disease patients. Anti-nuclear antibodies (ANA) had previously been present in 21% of PSC patients, 63% of AI-CAH patients and 48% of PBC patients. Anti-mitochondrial antibodies (AMA) had been found in 95% of PBC patients and not in any other patient. Also, 46% of PSC patients and 13% of AI-CAH patients suffered from UC.
Further controls included 252 healthy blood bank donors (137 men and 115 women; median age = 49 yr; range = 18 to 65 yr) and sera from patients with proteinase 3, myeloperoxidase, elastase and lactoferrin antibodies.
We prepared $F(ab')_2$ fragments of all sera by treating the sera for 16 hrs at 37 °C with pepsin 0.66 mg/ml (Sigma Chemical Co., St. Louis, MO) in sodium acetate buffer 0.1 mol/L (pH 4.5).

Antibodies:
Monoclonal antibodies directed against proteinase 3 (12.8; Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands), myeloperoxidase (14.15; CLB), and elastase (M752; Dakopatts, Copenhagen, Denmark) and polyclonal antibodies directed against lactoferrin (A186; Dakopatts) were used.

Indirect Immunofluorescence:
Detection of ANCA was done as described before (15). In brief, purified granulocytes from a healthy donor were applied on Cooke multiwell slides (Nutacon, Schiphol-Oost, The Netherlands) at a concentration of 1 x 10^6 cells/ml. The slides were left for 15' at 37 °C, during which time the cells adhered to the slides. After ethanol fixation for 10 min at 4 °C, the slides were air-dried and stored at -80 °C until use. After being wetted with PBS, 1:16 to 1:512 serial dilutions in PBS of test or control sera were applied to the wells. After incubation for 1 hr at room temperature, the slides were washed three times with PBS, and bound antibodies were detected with FITC-conjugated $F(ab')_2$ fragments of rabbit antibodies to human IgG, diluted 1:100 (F315; Dakopatts). Subsequently, the slides were washed three times in PBS and covered with glycerin PBS. Slides were read by two independent observers who were unaware of the clinical diagnosis. A titer greater than 1:32 was considered positive.
To investigate whether ANCA in the test sera recognized cytoplasmic antigens, we also performed detection of ANCA on paraformaldehyde fixed cells. Cytocentrifuged granulocytes were fixed in 0.5% phosphate-buffered paraformaldehyde (pH 8.5) for 10 min at room temperature, after which detection of ANCA was performed as described above.
Detection of ANA was performed on ethanol fixed HEp2 cells, identical to methods described above for ANCA. The patients were not tested for liver-kidney microsomal antibodies or soluble liver antigen antibodies.

Elisa:
As previously described (4, 16), we used an antigen capture Elisa to test sera for the presence of either proteinase 3, myeloperoxidase, or human leukocyte elastase antibodies. As a conjugate alkaline phosphatase-conjugated $F(ab')_2$ fragments of human IgG antibodies were used (A5403; Sigma Chemical Co.), diluted 1:500. The presence of anti-lactoferrin antibodies was detected by Elisa on plates directly coated with lactoferrin (Serva, Heidelberg, Germany), at a concentration of 7 μg/ml in bicarbonate buffer 0.1 mol/L (pH 9.6). Peroxidase conjugated rabbit antibodies to human Ig, diluted 1:500, were used for detection (P212, Dakopatts). Results by Elisa were considered positive when the values obtained exceeded the mean of 50 normal control sera by more than 3 SD.

Western blotting:
SDS-PAGE was performed according to the method of Laemmli. The neutrophil suspension (2 x 10^8 cells/ml) was sonicated three times for 20 sec in NaCl 1 mol/L containing PMSF 5 mmol/L (Sigma Chemical Co.). Membrane fragments were removed by ultracentrifugation for 2 hr at 100,000 g, and an equivalent of 6 x 10^7 cells was applied on 10% polyacrylamide gel under denaturing but non-reducing conditions. After electrophoresis and transfer to nitrocellulose (Schleicher and Schuell, Keene, NH) the blots were blocked overnight with 10% horse serum (CLB). Serum samples were applied at a dilution of 1:50 in Tris-HCl 0.01 mol/L, containing NaCl 0.15 mol/L, 0.1% Triton-X100 and 1% horse serum for 2 hr. After extensive washing, bound antibodies were detected with peroxidase-conjugated antibodies to human IgG (P214; Dakopatts). The reaction was visualized with 4-chloro-1-naphtol (Sigma Chemical Co.) and H_2O_2 as a substrate. Molecular weight standards (Bio-Rad low and high range; Biorad Laboratories Ltd., Richmond, CA) were used as references.
A cellular extract of HEp2 cells was prepared following exactly the same procedure.

Statistics:
Statistical analysis was performed using the χ^2 test.

Results

Detection of ANCA by IIF:
ANCA were detected by IIF on ethanol-fixed granulocytes in 19 of 24 sera from PSC patients (79%), 21 of 24 sera from AI-CAH patients (88%), and 7 of 25 sera from PBC patients (28%). ANCA patterns were invariably p-ANCA (figure 1). Median titers of the ANCA positive sera were 128 (range = 32 to 512) for PSC, 256 (range = 32 to 512) for AI-CAH, and 128 (range = 32 to 512) for PBC (figure 2).The presence of ANCA was not related to the use of immunosuppressive drugs, and patients taking immunosuppressive drugs did not show significantly lower ANCA titers. Although atypical staining of the neutrophil cytoplasm and nucleus could be observed in 30% of the sera, p-ANCA were not detected in other chronic liver diseases (n=23) (table I). This atypical staining disappeared after pepsin treatment of the sera, whereas p-ANCA patterns were still produced by pepsin treated PSC, AI-CAH and PBC sera.

p-ANCA were also detected in 12 of 252 (5%) healthy blood bank donor sera. The median titer of ANCA in the positive sera was 64 (range = 32 to 512).

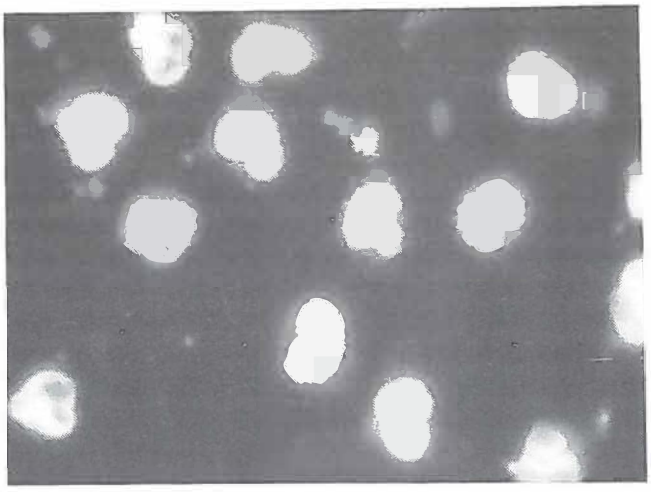

Figure 1. p-ANCA pattern on ethanol fixed neutrophils.

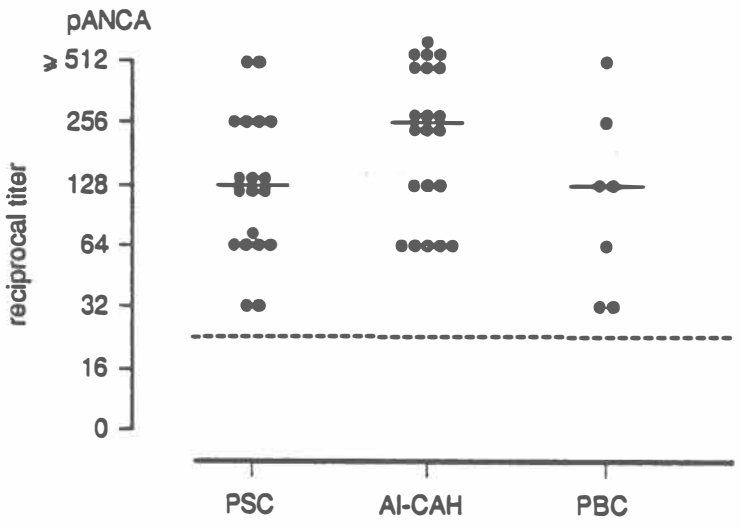

Figure 2. p-ANCA titers as determined by IIF on ethanol fixed granulocytes. Median titers are represented by solid lines; discrimination between positive and negative is represented by the dashed line.

To evaluate the cytoplasmic nature of the antigens recognized by p-ANCA, we tested all sera by IIF on paraformaldehyde fixed neutrophils. All p-ANCA positive sera from PSC-, AI-CAH- and PBC-patients displayed a cytoplasmic fluorescence pattern on paraformaldehyde fixed cells (figure 3). In addition, three sera from PBC patients and one serum from a PSC patient who had scored negative for p-ANCA on ethanol fixed neutrophils, showed cytoplasmic staining in this test. The sera that produced atypical staining of ethanol fixed neutrophils did not produce staining of paraformaldehyde fixed cells.

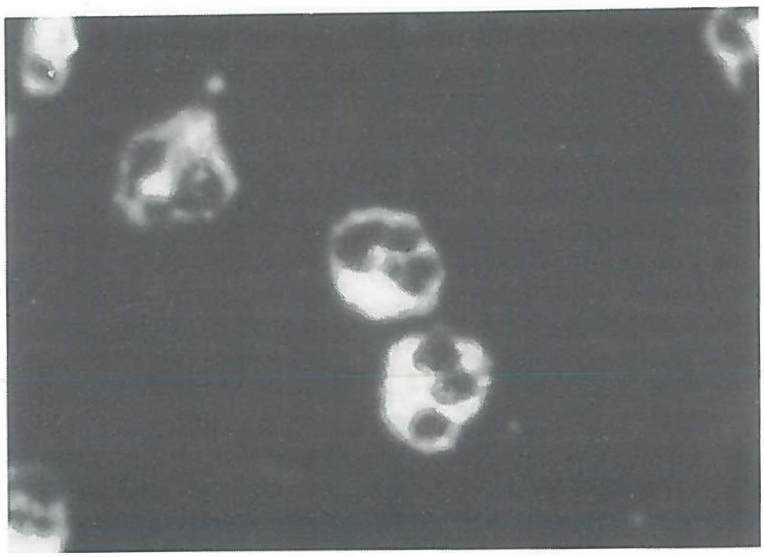

Figure 3. Diffuse cytoplasmic staining of a p-ANCA containing serum on paraformaldehyde fixed neutrophils.

Characterization of ANCA by Elisa:

All sera from patients with PSC, AI-CAH and PBC were tested for reactivity to proteinase 3, myeloperoxidase, elastase or lactoferrin. One p-ANCA positive serum from a patient with PBC was positive for antibodies to myeloperoxidase. Five of 19 p-ANCA positive PSC sera (26%), and 2 of 21 p-ANCA positive AI-CAH sera (10%) reacted with lactoferrin. All other sera were negative for either proteinase 3, myeloperoxidase, elastase or lactoferrin.

Characterization of ANCA by Western blotting:

Next, all sera were tested for reactivity with neutrophil components by Western blotting. Reactivity was observed with either lactoferrin, a 67/66 protein doublet or a 40 kD polypeptide in 18 sera (figure 4). Fifteen of these sera were positive for ANCA by IIF on neutrophils fixed with ethanol or paraformaldehyde; three were negative by IIF. The frequency of reactivity with these proteins within the three disease populations is given

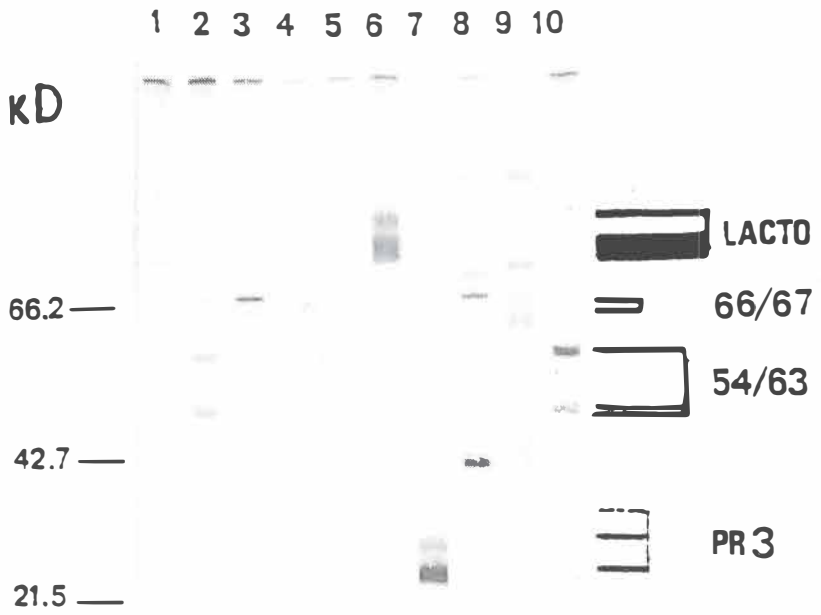

Figure 4. Western blotting with sonicated neutrophils as antigenic substrate; lane 1, buffer control; lane 2, IBD serum; lane 3, PBC serum; lane 4, IBD serum; lane 5, negative control; lane 6, PSC serum; lane 7, proteinase 3 MoAb; lane 8, PSC serum; lane 9, myeloperoxidase polyclonal antibody; lane 10, IBD serum.

Table II. Numbers of sera from patients with autoimmune liver diseases reacting with neutrophil proteins as detected by Western blotting.

Proteins	PSC[1] (n=24)	AI-CAH[1] (n=24)	PBC[1] (n=25)
lactoferrin	4	2	0
67/66 kD	4[2]	2	4[3]
40 kD	1	0	1
total (%)	9 (38%)	4 (17%)	5 (20%)

[1] PSC: primary sclerosing cholangitis, AI-CAH: autoimmune chronic active hepatitis, PBC: primary biliary cirrhosis.

[2] two sera did not show reactivity with ethanol fixed cells by IIF, one of those produced cytoplasmic fluorescence on paraformaldehyde fixed cells.

[3] one serum did not react with ethanol or paraformaldehyde fixed cells by IIF.

in table 2. Reactivity was found in 9 of 24 PSC patients (38%), 4 of 24 AI-CAH patients (17%) and in 5 of 25 PBC patients (20%). Four of the five PSC sera and the two AI-CAH sera that were positive for lactoferrin antibodies by Elisa reacted with lactoferrin on blot as well. Positive controls (i.e., ANCA directed against proteinase 3, myeloperoxidase or human leukocyte elastase) showed reactivity with bands of the expected sizes, and negative controls (i.e., sera from bloodbank donors) did not show reactivity on blot. Sera positive in Western blotting with a neutrophil extract as a substrate did not react with proteins of comparable sizes when a cellular extract of HEp2 cells was used as substrate.

Clinical and serological associations of ANCA in autoimmune liver diseases:

No relation could be observed between the presence of ANCA and either transaminase levels or duration of disease. However, the presence of ANCA did correlate significantly ($p < 0.008$) with the severity of the disease as assessed by the presence of cirrhosis (figure 5).

The presence of ANCA was not associated with the concomitant occurrence of UC. Although PSC is often associated with UC, ANCA were not restricted to patients with UC (10 out of the 19 p-ANCA positive patients with PSC did not have UC), and ANCA did not always occur in patients with UC (2 out of the 11 patients with PSC and UC were negative for p-ANCA).

ANA were detected in 4 of the PSC sera (17%), 9 of the AI-CAH sera (38%) and in 10 of the PBC sera (40%) at the time of serum sampling for determination of ANCA. Occurrence of ANCA did not correlate with a positive test for ANA (7 out of 19 sera with a positive test for ANA were p-ANCA negative).

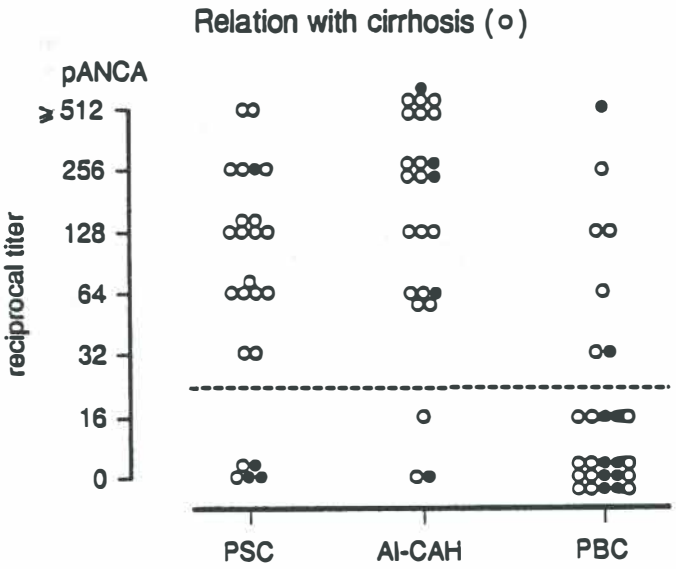

Figure 5. Presence and titers of p-ANCA in PSC (n=24), AI-CAH (n=24), and PBC (n=25) in relation to the concomitant presence (open circles) or absence (closed circles) of cirrhosis.

Discussion

After the detection of p-ANCA in a substantial number of sera from patients with UC (7-11), these autoantibodies were reported to occur also in PSC (8,12,13), an autoimmune liver disease frequently associated with UC. We here report that ANCA are present in 79% of the PSC sera, but also in 88% of AI-CAH sera and in 28% of PBC sera. All ANCA detected produced a perinuclear fluorescence pattern on ethanol fixed neutrophils and a diffuse granular cytoplasmic staining on paraformaldehyde fixed cells. Sera from patients with non-autoimmune chronic liver diseases did not contain ANCA, although they sometimes produced atypical staining of the neutrophils. This atypical staining disappeared after pepsin treatment of the sera, whereas p-ANCA patterns were still produced by pepsin treated PSC, AI-CAH and PBC sera. Titers of ANCA were comparable in the three autoimmune liver diseases.

The prevalence of ANCA in PSC as found in our patients is in accordance with that found by other investigators (9,12,13). Our study, however, shows a much higher prevalence of ANCA in AI-CAH than in other reports (12,13,17-19). This finding might be a result of the method used for their detection. Klein et al.(12), who found p-ANCA in 16% of their AI-CAH patients, used methanol fixed neutrophils as a substrate. Previous studies from our laboratory have shown that granulocyte specific ANA in rheumatoid arthritis (RA), which have been shown to recognize neutrophil cytoplasmic antigens (and as such can be considered as ANCA (20)), react with ethanol fixed neutrophils but are unreactive after methanol fixation of the substrate (Mulder AHL et al. manuscript submitted, 1992). This finding might also be the case for ANCA in AI-CAH. Smalley, Mackay and Whittingham (17) reported a 50% prevalence of GS-ANA in AI-CAH, and Wiik (18) found GS-ANA to occur incidentally in AI-CAH. Both authors used the classic definition of GS-ANA: antibodies that show enhanced reactivity with nuclei of neutrophils compared to their reactivity with nuclei of other cells (i.e., at least a two titerstep difference). Because ANA are frequently reported in sera of AI-CAH patients, sera containing both ANCA and high-titered ANA are not scored positive for ANCA when this definition is used. The lower prevalence of ANCA (or GS-ANA as these antibodies were designated at that time) in AI-CAH, as reported in those studies compared to our results, might be explained by the definition used for GS-ANA/ANCA.

The prevalence of ANCA in PBC patients seems to be much lower than that in the PSC and AI-CAH populations. The number of ANCA-positive sera in patients with less advanced disease (i.e., without cirrhosis) was significantly lower than that in cirrhotic patients, and although fewer patients with cirrhosis were included in the PBC group compared to the other two autoimmune liver diseases, the lower prevalence of ANCA in PBC can not be totally attributed to the lower cirrhosis incidence.

The p-ANCA pattern produced by the sera proved to be caused by artificial redistribution of the antigen: IIF of the sera on paraformaldehyde fixed cells resulted in diffuse granular cytoplasmic staining. Additionally, we showed that the perinuclear staining of the neutrophils was not produced by ANA, since far less ANA were detected in these sera than were ANCA. We conclude that ANCA in autoimmune liver diseases recognize cytoplasmic antigens.

ANCA in autoimmune liver disease do not recognize either proteinase 3, myeloperoxidase or elastase, the three antigens that are recognized by ANCA in Wegener's granulomatosis and systemic vasculitides (4,5,16). On Western blot we found reactivity of the autoimmune liver disease sera with lactoferrin, a yet uncharacterized protein doublet of 67/66 kD, or an uncharacterized protein of 40 kD. Of all p-ANCA positive sera, 37% of PSC sera, 43% of PBC sera and only 17% of AI-CAH sera could be identified by Western blotting to react with one of these proteins. Thus, although ANCA reacting with lactoferrin, the 67/66 kD doublet or the 40 kD polypeptide can be found in all three autoimmune liver diseases, prevalence of these specificities differ among the three diseases. Interestingly, reactivity with lactoferrin and the 66/67 kD doublet was not restricted to ANCA in autoimmune liver diseases but was also observed for ANCA in UC and Crohn's disease (see figure 4) and for ANCA in RA (20, 21). In contrast to the specificity of proteinase 3/c-ANCA antibodies for Wegener's granulomatosis, p-ANCA reacting with lactoferrin or the 66/67 kD doublet are not specific for one disease but might in fact be a marker of chronic inflammation in an autoimmune environment.

A number of p-ANCA positive sera in autoimmune liver diseases are directed against lactoferrin, which we demonstrated both by Elisa and Western blotting. We found that other p-ANCA positive sera react with yet uncharacterized polypeptides of 66/67 kD and 40 kD. Klein et al.(12) and Seibold et al.(13), studying reactivity of sera from PSC patients by Western blotting, found these sera to react with unknown determinants of 90 kD, 60 kD, 55 kD, 40 kD, and 30 kD proteins, using essentially the same methods as we did. Reactivity with these determinants was highly specific for PSC and was not found in IBD or AI-CAH. This contrasts with our finding that reactivity with a number of polypeptides occurred as well in IBD and RA. Although the 40 kD polypeptide that they describe might be identical to the 40 kD polypeptide in our study, controversy still exists about the antigenic specificities of p-ANCA in autoimmune liver diseases, and further characterization studies have to be performed.

ANCA have now been reported in a broad spectrum of autoimmune diseases, all characterized by chronic inflammation, such as RA, UC and PSC. As such, no disease specificity can be assigned to p-ANCA of as yet undefined antigenic specificity, in contrast to c-ANCA, which is highly specific for Wegener's granulomatosis (4,22) and p-ANCA directed against myeloperoxidase, which is associated with idiopathic necrotizing systemic vasculitis and glomerulonephritis (4, 5). The pathogenetic role for ANCA in autoimmune liver disease, however, is still unclear. Whereas a rise in ANCA titer is observed prior to relapses of Wegener's disease (22) and c-ANCA can be found in early Wegener's disease, ANCA in autoimmune liver disease are especially associated with cirrhosis and probably appear later on in the course of the disease. Possibly, these autoantibodies are an epiphenomenon of chronic inflammation. Whether the antibodies to lactoferrin and the still uncharacterized proteins in autoimmune liver diseases, RA and IBD are just an epiphenomenon of inflammation or whether they maintain the inflammatory process by inducing both degranulation and respiratory burst of primed neutrophils in vivo (23) has to be established.

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CHAPTER 3.2

NEUTROPHIL CYTOPLASMIC AUTOANTIBODIES AFTER LIVER TRANSPLANTATION IN PATIENTS WITH PRIMARY SCLEROSING CHOLANGITIS.

Haagsma EB, Mulder AHL, Gouw ASH, Horst G, Meerman L, Slooff MJH, and Kallenberg CGM.

Summary

The immunopathogenic importance of neutrophil cytoplasmic autantibodies in ulcerative colitis and primary sclerosing cholangitis is unknown. These autoantibodies were investigated before and after liver transplantation in 9 patients with primary sclerosing cholangitis. Sera from 10 patients transplanted for metabolic disorders or hemangioma served as controls. Before liver transplantation neutrophil cytoplasmic autoantibodies, producing a perinuclear pattern by indirect immunofluorescence on ethanol fixed neutrophils, were present in all patients with primary sclerosing cholangitis. A decline in titer was noted in the first months after liver transplantation. During longterm follow up, the autoantibodies remained present and most often the titer did not differ from before transplantation. They were not directed against proteinase 3, myeloperoxidase, elastase, or lactoferrin. All but one of the control patients were negative for the autoantibody. No relation was seen, before or after transplantation, with ulcerative colitis or proctocolectomy. There was no recurrence of primary sclerosing cholangitis in any of the patients as judged by liver histology. We conclude that neutrophil cytoplasmic autoantibodies remain present after liver transplantation for primary sclerosing cholangitis and that its synthesis is not related to the presence of the diseased organ(s). The primary disease process in primary sclerosing cholangitis and ulcerative colitis may well be a disturbance of the immune system.

End-stage primary sclerosing cholangitis (PSC) can be treated by orthotopic liver transplantation. During this operation the whole biliary tree is removed with the exception of the most distal part of the choledochal duct. Many patients with PSC have co-existing ulcerative colitis and some have undergone colectomy prior to liver transplantation. After liver transplantation periods of exacerbation of ulcerative colitis have been shown to occur in about one third of patients (1). Whether PSC may recur after transplantation is still a matter of debate (1-4).

Autoantibodies to neutrophils producing a perinuclear pattern by indirect immunofluorescence on ethanol fixed neutrophils (p-ANCA), have been detected in patients with PSC (5) and ulcerative colitis (5,6). The cytoplasmic localization of the antigens recognized by these autoantibodies has been demonstrated by their cytoplasmic staining of neutrophils fixed with para-formaldehyde (7). The nature of the antigen(s) recognized by p-ANCA in these aforementioned conditions, however, has not been elucidated so far, although reactivity with lactoferrin is found in a number of PSC and ulcerative colitis sera (7). Whether these autoantibodies are of immunopathogenic importance is still unknown. Also, the influence of the diseased organ on the regulation of the autoantibody synthesis has not been clarified. Studying serial levels of p-ANCA in patients with PSC who have undergone an orthotopic liver transplantation might clarify some of these issues.

The presence of neutrophil cytoplasmic autoantibodies was investigated in 9 patients with PSC before and after transplantation. A relation was looked for between changes in p-ANCA titers after transplantation and the presence of the possible recurrence of liver disease and/or inflammatory bowel disease. To assess the specificity of the autoantibody response, serial changes were assessed in titers of p-ANCA in relation to changes in total levels of immunoglobulin G and antibodies to tetanus toxoid, a recall antigen not related to ANCA.

Patients

Since 1986 9 patients with PSC have been transplanted, 4 men and 5 women, age median 32 years (range 23-45). Preoperative patient characteristics are listed in Table 1. Patient No 1. was retransplanted after 8 months because of intrahepatic abscesses and necrotic bile ducts due to a thrombosed hepatic artery. He died 6 months later of metastasis of a hepatocellular carcinoma in the original PSC liver. For the present study he was followed until retransplantation. Patient No 2. developed chronic rejection. He died of pneumocystic carinii infection while waiting for a retransplant 4 months after transplantation. The other 7 patients are alive 3 months to 4 years after transplantation.

In all but one patient the diagnosis of PSC was made pre-operatively with appropriate clinical, biochemical, histochemical and endoscopic cholangiographic criteria (8). In all cases the diagnosis was confirmed by histologic examination of the removed liver.

On referral for liver transplantation 3 patients had a history of ulcerative colitis. Patient No. 4 developed ulcerative colitis 3 years before transplantation and was succesfully treated with salazopyrine until transplantation. Patient No. 7 developed ulcerative colitis 26 years before transplantation. She underwent a proctocolectomy with ileostoma 6 years before transplantation due to serious dysplasia and colloid carcinoma of the colonic

mucosa as detected by control coloscopy. Patient No. 9 developed ulcerative colitis 9 years before liver transplantation, she was also treated succesfully with salazopyrine, and later on with pentasa until transplantation. The other 6 patients did not have a history of ulcerative colitis. During the work-up for liver transplantation 5 of these 6 patients underwent sigmoidoscopy and biopsies. No signs of colitis were found.
Transfusion requirements during liver transplantation were median 5 liters, range 2-16 liters.

Table I. Characteristics of nine patients with primary sclerosing cholangitis who underwent liver transplantation

Patient no.	sex	age at OLT (yr)	Follow-up after OLT (months)	History of ulcerative colitis
1	M	25	8	negative
2	M	25	4	negative
3	F	28	48	negative
4	M	45	39	positive, salazopyrine
5	F	41	32	negaative
6	M	32	30	negative
7	F	40	25	positive, proctocolectomy
8	F	23	24	negative
9	F	37	3	positive, salazopyrine/-pentasa

After transplantation immunosuppression consisted of prednisolone, azathioprine and cyclosporine A. After 1, 2, 3 and 6 months the median prednisolone dosage was 25, 20, 19 and 17 mg/day, respectively. After 1, 2, 3 and 4 years the median dosage amounted to 13, 10, 11 and 10 mg/day, respectively. Azathioprine, 125-150 mg/day, was started from day 1 onward. Whole blood cyclosporine A through levels aimed at were 200-250 ng/ml in the first 3 weeks, afterwards 100-150 ng/ml.
According to our protocol a liver biopsy was done every whole year after liver transplantation.

Sera

Freeze stored sera obtained before (within one month) and after transplantation were investigated. From all nine patients sera were studied before and 1, 2, and 3 months after transplantation, respectively. In addition, sera were studied at 6, 12, 18, 24, 30, 36, 42, and 48 months from 7, 6, 6, 6, 2, 2, 1, and 1 patients, respectively.

Control patients

Sera from 10 patients were studied who underwent liver transplantation for diseases of non-autoimmune pathogenesis were studied as controls, i.e. Budd Chiari's syndrome

(n=4), hemangioma (n=2), erythropoietic protoporphyria (n=2), alpha-1-antitrypsin deficiency (n=1) and Wilson's disease (n=1). One serum sample was investigated from each patient for the presence of p-ANCA. These sera were taken median 3 years (range 1-9) after transplantation.

Methods

Indirect immuno-fluorescence

Testing for ANCA was done according to Van der Woude et al. (9). Briefly, granulocytes from a healthy donor were obtained by Isopaque-Ficoll centrifugation and dextran sedimentation of EDTA blood. After hypotonic lysis of the erythrocytes and washing of the granulocytes, cytocentrifuge preparations were made and the air-dried slides were fixed with absolute ethanol (10', 4 °C). After wetting with PBS, test or control sera were applied in a dilution ranging from 1:16 to 1:512 in PBS. Following incubation for 1 hr at room temperature the slides were washed three times with PBS and bound antibody was detected with fluorescein isothiocyanate (FITC) conjugated $F(ab')_2$ fragments of rabbit-anti-human IgG, diluted 1:100 (Dakopatts, Copenhagen). The slides were subsequently washed three times in PBS and covered with glycerin-phosphate buffered saline. Slides were read by two independent observers, unaware of the clinical diagnosis.
Control sera included sera from healthy donors and from patients with ANCA of defined specificity: anti-myeloperoxidase (α-mpo), anti-proteinase 3 (α-pr3), anti-elastase (α-hle) and anti-lactoferrin (α-lf) or from patients with defined antinuclear antibodies (ANA): anti-ds DNA, anti-ENA (Sm, U1-RNP, SS-A and SS-B) and anti-histone. Testing for ANA occurred by indirect immunofluorescence on ethanol fixed HEp2 cells.

Elisa

An antigen capture Elisa (10) was used to test sera for the presence of either anti-PR3, anti-MPO or anti-HLE antibodies. Conjugate used was anti-human IgG alkaline phosphatase conjugated $F(ab')_2$ antibody (A5404, Sigma, St. Louis), diluted 1:500.
The presence of anti-LF antibodies was detected by Elisa in which lactoferrin (Serva, Heidelberg) was coated directly at a concentration of 7 μg/ml. Peroxidase conjugated rabbit Ig to human total Ig (P212, Dakopatts, Copenhagen), diluted 1:500 was used for detection. Anti-tetanus antibodies were measured by Elisa according to routine procedures (11).

Total IgG

Total IgG was measured by nephelometry. Normal values in our laboratory ranged from 8.5-15 g/l.

Results

Before liver transplantation p-ANCA were present in all PSC patients with a median titer

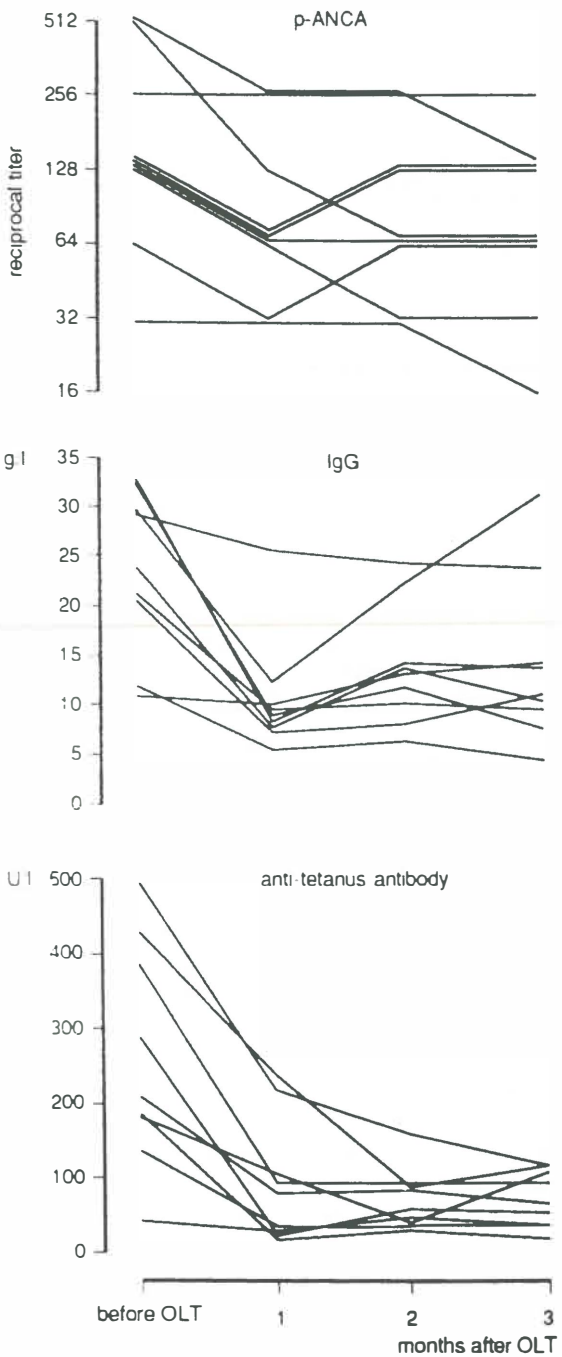

Figure 1. Decline of p-ANCA titers, IgG and anti-tetanus antibody levels during the first 3 months after liver transplantation for primary sclerosing cholangitis

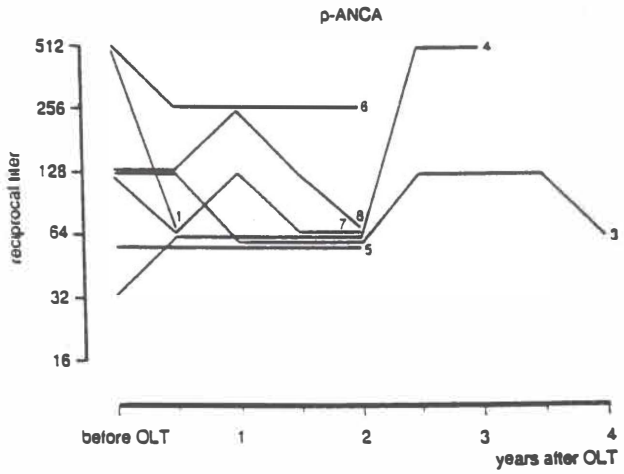

Figure 2. Follow-up of p-ANCA titers in 7 patients with primary sclerosing cholangitis with a survival > 6 months after liver transplantation. * Patients number according to Table 1.

of 128 (range 32-512). No differences in titer were seen between PSC patients with or without a history of ulceraive colitis. A decline in titer was seen in the first 3 months after transplantation in concurrence with a decline in IgG and anti-tetanus concentrations (figure 1).

More long-term, p-ANCA remained present in all patients, irrespective of a preoperative history of ulcerative colitis or proctocolectomy, and, with the exception of patient No. 4, the titer did not differ significantly from the preoperative value (figure 2). After transplantation the IgG and anti-tetanus levels were lower than before transplantation but did not show much variation in the long-term.

p-ANCA was not directed against defined cytoplasmic antigens i.e. myeloperoxidase, elastase, proteinase 3 or lactoferrin in any of the patients before or after liver transplantation.

ANA were present in only 3 patients (Nos. 4, 5, and 7). These ANA were in a low titer, and showed a diffusely speckled, nucleolar, and a nucleolar pattern on a homogenous background, respectively. After transplantation no significant changes in titer were noted.

The sera from the 10 control liver transplant patients were negative for p-ANCA, except for one serum. This serum was derived from a patient who acquired a chronic hepatitis B infection in the first year after liver transplantation. The titer was low (1:32).

An exacerbation of ulcerative colitis was only seen in one out of the 3 patients with ulcerative colitis before liver transplantation (patient No. 4). Its relation to levels of p-ANCA is described below. In the 6 patients without a history of ulcerative colitis before transplantation, colitis did not develop newly after transplantation.

Recurrence of PSC was not found in the protocol liver biopsies performed once every year after liver transplantation. Liver histology was normal in patients Nos. 3, 5 and 7. In

year after liver transplantation. Liver histology was normal in patients Nos. 3, 5 and 7. In patients Nos. 4 and 6 a slight mononuclear infiltrate without bile duct damage was seen in some portal tracts with mild portal fibrosis in patient No. 4 (see case history below). Rejection grade 1 out of 4 (12) with endothelialitis and bile duct damage was seen in the one year biopsy of patient No. 8 which resolved later on. Concentric periductular fibrosis or non-suppurative destructive cholangitis (without endothelialitis), both suggestive of recurrence of PSC, were not seen.

Case history of patient No. 4

Patient No. 4 (figure 3) had a history of ulcerative colitis before liver transplantation. In the 6th month after transplantation he developed an exacerbation of ulcerative colitis which was treated successfully with pentasa and prednisolone enemas. Although IgG and anti-tetanus levels were significantly lower at this time than before transplantation, the p-ANCA titer remained unchanged compared to the pre-transplant liver. After the second year it was decided to discontinue the cyclosporine medication in this patient because of expected longterm side-effects on renal function. Prednisolone and azathioprine were continued. The one and two year liver biopsies showed slight mononuclear infiltrate in some portal tracts without bile duct damage, with, in addition, mild portal fibrosis in the 2-year biopsy. Liver tests became abnormal after this without jaundice and remained abnormal even after an increase of the prednisolone dosage. The p-ANCA titer increased significantly. In contrast, the ANA titer and anti-tetanus level did not change and the IgG level only slightly increased. Repeated investigations by ultrasound never indicated bile duct obstruction. ERCP was preculuded by the presence of a hepaticojejunostomy with Roux-Y deviation. Histologically the liver biopsy taken 32 months after transplantation was not different from the histologic findings at 2 years. No sighns of rejection or recurrence of disease were found.

Discussion

p-ANCA have been demonstrated in sera from patients with ulcerative colitis and PSC (5, 6). In the present study all 9 patients with PSC had p-ANCA before liver transplantation. The target antigen(s) of p-ANCA in ulcerative colitis and PSC have not yet been identified (5, 6), although recently reactivity with multiple unidentified proteins by Western blotting have been described particularly in sera from PSC patients (13). In the present study p-ANCA did not react with proteinase 3, myeloperoxidase, elastase or lactoferrin. In a previous study it was shown that in some patients p-ANCA react with lactoferrin (7).The role of p-ANCA in PSC and ulcerative colitis is unclear. It is unknown whether they are involved in the immunopathogenesis of these diseases, reflect a specific defect in im-muoregulation or are immunologic epiphenomena of diagnostic value.

In the present study it was found that p-ANCA titers initially decreased after liver transplantation, which may be attributed to the high dose immunosuppression needed. Likewise, the total IgG and the anti-tetanus antibody levels decreased. An alternative or

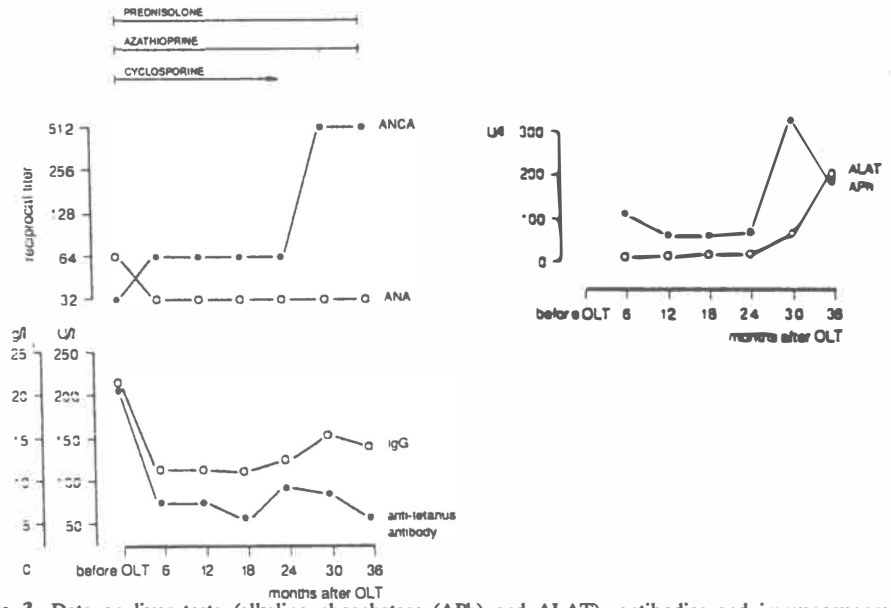

Figure 3. Data on liver tests (alkaline phosphatase (APh) and ALAT), antibodies and immunosuppression of patient No. 4 with a follow-up of 3 years after liver transplantation.

additive explanation may be a dilutional effect due to the preoperative transfusion requirements.

The production of p-ANCA seems to be independent of the presence of a disease liver or colon. Up to 4 years after liver transplantation, p-ANCA remained present in most cases in titers which were not different from before transplantation. The presence of p-ANCA after liver transplantation is not related to liver transplantation as such since p-ANCA were absent in all but one of the 10 control patients. It has already been reported that p-ANCA persist after colectomy in patients with ulcerative colitis (6). In addition, this study included one patient who had both diseased organs, i.e. colon and liver, removed, and p-ANCA still was present. Recently a 40 kD colonic protein has been described that specifically reacts with IgG eluted from colonic specimens of patients with ulcerative colitis (14). A monoclonal antibody raised against this protein also reacted with skin epithelial cells and mucosa epithelial cells from the gall bladder, bile duct and hepatic ducts (15). Although this monoclonal antibody was not tested on human neutrophils cross-reactivity with myeloid cells seems unlikely. In addition, p-ANCA positive sera do not react with bile duct epithelium in liver sections (16). These and the present findings suggest that the diseased organs are not involved in the antigen-specific stimulation of p-ANCA producing clones.

A comparison between PSC and primary biliary cirrhosis can now be made. In primary biliary cirrhosis after transplantation the antimitochondrial antibodies (AMA) and their disease specific subtyped remain present (17), which is compatible with the notion this disease is in essence a systemic disease. Whether the primary biliary cirrhosis recurs after transplantation is still a matter of debate (17-20) and whether AMA plays a role in this

respect is not yet clarified. The same can be stated of PSC and p-ANCA. Recurrence of PSC after transplantation has so far not been proven (2-4). In this respect some difficulties are encountered. First, in non-transplant patients the diagnosis of PSC depends heavily on typical features seen on cholangiography performed by ERCP. Histological features of bile duct damage and periductular concentric fibrosis help make the diagnosis (21). After liver transplantation, however, usually the biliary tree cannot be easily visualized by ERCP whereas almost all patients receive a choledochojejunostomy with Roux-Y deviation. In these cases percutaneous transhepatic cholangiography is needed for visualization of the biliary tree. This technique is however more invasive, not always easy in the absence of bile duct dilatation and carries an increased risk of septic complications probably due to the colonization of the biliary tree with gastrointestinal flora in immunosuppressed patients (22). Second, the cholangiographic features of sclerosing cholangitis are also seen in chronic rejection (23). Also, like PSC chronic rejection is histologically characterized by bile duct damage. Therefore, the demonstration of recurrence of PSC after liver transplantation will mainly depend on comparison of PSC and non-PSC groups. The primary aim of the present study was the follow-up of autoantibodies after liver transplantation. Liver histology was not investigated in a controlled way since the number of patients was still too small and the follow-up probably too short to come to definite conclusions as to the recurrence of PSC. Still it can be said that none of the liver biopsies up to 4 years after transplantation showed bile duct damage other than due to acute rejection. One patient developed cholestatic liver test disturbances without histologic signs of rejection and without signs of bile duct obstruction as judged by ultrasound. An ERCP could not be performed. In the differential diagnosis of this case sclerosing cholangitis was considered due to either mild chronic rejection or the recurrence of PSC. With respect to the latter it is of interest to note that this was the only patient who had a relapse of colitis and increase in p-ANCA titers to above pre-transplant values. Future follow-up will show whether the increase in p-ANCA titer in this patient was an indication of a recurrence of PSC.

In Wegener's granulomatosis, ANCA is directed against the myeloid lysosomal enzyme proteinase 3, and relapses of disease have been shown to be preceeded by recurrence or increase of ANCA levels, thus suggesting immunopathogenic importance (24). In ulcerative colitis, transversal studies did not show a relation between p-ANCA titer and disease activity in some reports (5, 6, 25), although other described different results (16). However, longitudinal studies are needed to confirm this relation. It may be concluded from the present study and the studies mentioned above, that p-ANCA do not seem to play an immunopathogenic role in the development of PSC and ulcerative colitis.

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CHAPTER 4.1

ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA) IN SERA FROM PATIENTS WITH INFLAMMATORY BOWEL DISEASE (IBD): RELATION TO DISEASE PATTERN AND DISEASE ACTIVITY.

J. Broekroelofs, A.H.L. Mulder, G.F. Nelis, B.D. Westerveld, J.W. Cohen Tervaert and C.G.M. Kallenberg.

Summary

Anti-neutrophil cytoplasmic antibodies producing a perinuclear fluorescence pattern on ethanol-fixed granulocytes (p-ANCA) were found in 33 out of 67 patients (49%) with ulcerative colitis (UC) but also in 14 out of 35 patients (40%) with Crohn's disease (CD). In the latter condition p-ANCA were equally present in subgroups with colonic, ileocolonic or ileal involvement only. Titers of p-ANCA were higher in patients with UC compared to CD-patients, in particular when comparing patients with active disease. In contrast to findings in CD, patients with active UC had higher titers of p-ANCA than patients with inactive UC. Although p-ANCA were incidentally directed to lactoferrin, both in UC and CD, and to proteinase-3 and myeloperoxidase, in UC only, the antigenic nature of p-ANCA could not be identified in most of the cases. We conclude that, within the spectrum of inflammatory bowel disease, the presence of p-ANCA is not specific for UC. When titers of p-ANCA are taken into account, the presence of high titered p-ANCA, however, suggests active UC.

Anti-neutrophil cytoplasmic antibodies (ANCA) were first described in Wegener's granulomatosis (1). These antibodies produce a characteristic cytoplasmic staining pattern on ethanol-fixed neutrophils (c-ANCA). Their target antigen has been elucidated as proteinase-3 (PR-3) (2-4). Later on, ANCA were described that produced a perinuclear fluorescence pattern on ethanol-fixed neutrophils (p-ANCA). In many of these p-ANCA positive sera the antibodies were directed to myeloperoxidase (MPO). Anti-MPO antibodies were shown to be associated with different forms of necrotizing systemic vasculitis and idiopathic necrotizing crescentic glomerulonephritis (5-7).
More recently, p-ANCA have been detected in the sera from patients with inflammatory bowel disease, in particular ulcerative colitis (UC) but also in a minority of sera from patients with Crohn's disease (CD). The prevalence of p-ANCA in UC, however, varies from 59% to 88% between several studies (8-11). Discrepancy also consists concerning the relation between the presence of ANCA and disease activity in UC (8,9). These discrepancies are probably related to patient selection, techniques used for the detection of ANCA, and differences in cut-off point for the establishment of a positive result in the ANCA immunofluorescence test. In most of these studies titers of ANCA were not taken into account when evaluating differences in the occurrence of p-ANCA between UC and CD. Since the antigen(s) recognized by ANCA in IBD are not yet known and p-ANCA as detected by indirect immunofluorescence occur also in autoimmune liver diseases, rheumatoid arthritis and other conditions (reviewed in 12) the clinical significance of a positive test for p-ANCA for UC is not yet fully established.
In the present study we evaluated the prevalence of ANCA in our population of patients with IBD. In addition, we investigated the relationship between the titer of ANCA and disease activity both in UC and CD. Finally, we evaluated the antigenic specificity of ANCA in IBD by performing ELISA's for antibodies against well-known myeloid proteins.

Methods

Patients

We investigated 102 patients with IBD, 67 patients with UC and 35 patients with CD. The diagnosis of UC or CD was based on accepted clinical and endoscopic criteria supported by radiological and pathological findings (13). In particular, endoscopy in CD had to show discontinuous lesions with skip areas and serpentigenous ulcers, and had to be supported by histopathology. Data about disease activity were collected retrospectively. CD disease activity was scored as active or inactive based on the Bristol simple index (14). Active disease was defined as a disease index ≥ 4 (14). UC disease activity was scored active or inactive on the basis of history, physical examination, laboratory results, endoscopy, radiology and the overall clinician's opinion (15). Active disease was subsequentely graded by endoscopic findings in which severity of the inflammation was graded 0-2, 2 being spontaneously bleeding, 1 being bleeding only after contact, 0 being non-bleeding (16).
The group of 35 patients with CD consisted of 18 men and 17 women, mean age 41, range 20 - 85 years. CD was localized in the ileum only in 12 patients, in the colon only

in 16 patients, and both in ileum and colon in 7 patients. Active disease was diagnosed in 15 patients, median disease activity score was 7, range 4-11.
The group of 67 patients with UC consisted of 40 men and 27 women, mean age 45, range 16 - 78 years. Active disease was diagnosed in 19 patients, median score at endoscopy was 2, range 0 - 2.
13 out of 35 patients with CD and 17 out of 67 patients with UC took immunosuppressive medication (prednisone, azathioprine, cyclosporin or a combination). The other patients took no medication or sulfasalazine or mesalazine only.
Control sera included sera from 252 healthy bloodbank donors (137 men, 115 women, mean age 49, range 18 - 65 years) and sera from patients with ANCA of defined specificity (anti-proteinase-3, anti-myeloperoxidase, anti-elastase and anti-lactoferrin antibodies).
Furthermore monoclonal antibodies directed against myeloperoxidase (14.15, Central Laboratory for the Bloodtransfusion Service, Amsterdam), proteinase 3 (12.8, Central Laboratory for the Bloodtransfusion Service, Amsterdam) and elastase (M752, Dakopatts, Copenhagen, Denmark) and polyclonal antibodies directed against lactoferrin (A186, Dakopatts, Copenhagen, Denmark) were used.

Indirect immunofluorescence

Detection of ANCA was done as described before (17). Briefly, purified granulocytes from a healthy donor were applied on Cooke multi-well slides at a concentration of 10^6 cells/ml. The slides were left for 15 minutes at 37 °C during which time the cells adhered to the slides. Following ethanol fixation (10 minutes at 4 °C) the slides were air-dried and stored at -80 °C until use. After wetting with PBS, test or control sera were applied in a dilution of 1:16 in PBS and diluted two-fold till 1:512. Following incubation for 1 hour at room temperature (RT) the slides were washed three times with PBS and bound antibodies were detected with fluorescein isothiocyanate (FITC) conjugated F(ab')2 fragments of rabbit-anti-human IgG, diluted 1:100 (F315, Dakopatts, Copenhagen, Denmark). Subsequently, the slides were washed three times with PBS and covered with glycerin-phosphate buffered saline. Slides were read by two independent observers, not aware of the clinical diagnosis. Fluorescence patterns were scored as cytoplasmic (c-ANCA) or perinuclear (p-ANCA). A titer of $\geq$ 1:32 was considered positive, as based on the results obtained from healthy controls (table 1).

Elisa

An antigen capture Elisa (2, 5) was used to test sera for the presence of either anti-proteinase-3, anti-myeloperoxidase or anti-elastase antibodies. As a conjugate alkaline-phosphatase conjugated F(ab')2 fragments of anti-human IgG were used (A5404, Sigma, St. Louis, USA) diluted 1:500. The presence of anti-lactoferrin antibodies was detected by ELISA directly coated with lactoferrin (Serve, Heidelberg, Germany) at a concentration of 7 ug/ml. Peroxidase conjugated rabbit anti-human Ig, diluted 1:500 was used for detection (Dakopatts, P212, Copenhagen, Denmark). Detection of anti-cathepsin G antibodies was performed in a similar way, using cathepsin G (Calbiochem, La Jolla, CA, USA) at a concentration of 1 ug/ml. A test was considered positive when its OD value exceeded the mean + 3 SD of normal controls.

Statistics

The results are given in absolute numbers and/or percentages. Statistical analysis was performed with Wilcoxon's rank test for unpaired observations and χ square test. A p-value less than 0.05 was considered statistically significant.

Results

The prevalence of ANCA in sera from patients with UC and CD, in relation to disease localization and medication used, and in controls is given in table 1.
P-ANCA were present in 14 out of 35 patients with CD (40%) and in 33 out of 67 patients with UC (49%), and c-ANCA were detected in one patient with UC only.
P-ANCA were detected in 8 out of 16 patients with colonic CD (50%), in 3 out of 12 patients with ileal CD (25%), and in 7 patients with ileocolonic CD (42%).
Immunosuppressive medication was taken by 13 out of 35 patients with CD of whom 5 had a positive p-ANCA (38%) and by 17 out of 67 patients with UC of whom 8 had a positive p-ANCA (47%). p-ANCA were detected in 12 out of 252 healthy controls (5%).

Table I. Prevalence of ANCA in sera from 67 consecutive patients with ulcerative colitis (UC) and 35 with Crohn's disease (CD) in relation to localization of the disease, disease activity, and medication used.

	number of patients with	
	p-ANCA	c-ANCA
Crohn's disease	14/35 (40%)	0/35 (0%)
- localization		
* colonic	8/16 (50%)	
* ileal	3/12 (25%)	
* ileocolonic	3/ 7 (43%)	
- medication		
* immunosuppressives	5/13 (38%)	
* others or none	9/22 (41%)	
- disease activity		
* active	5/15 (33%)	
* inactive	9/20 (45%)	
Ulcerative colitis	33/67 (49%)	1/67 (1%)
- medication		
* immunosuppressives	8/17 (47%)	
* others or none	25/50 (59%)	1/50 (2%)
- disease activity		
* active	12/19 (63%)	
* inactive	21/48 (44%)	1/48 (2%)
Healty controls	12/252 (5%)	0/252 (0%)

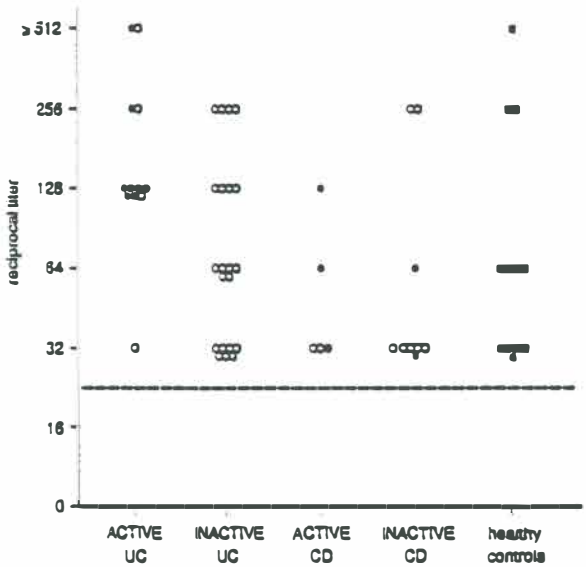

Figure 1. Titers of ANCA in patients with active and inactive ulcerative colitis (UC) and in patients with active and inactive Crohn's disease (CD). p-ANCA positive sera from patients taking immunosuppresives are represented by closed circles, while sera from patients taking other or no medication are represented by open circles. Healthy controls are represented by the squares. Discrimination between negative and positive result is represented by the dashed line.

Data about disease activity were collected retrospectively without knowledge of the results of ANCA-testing. The disease was scored as active in 15 patients with CD and in 19 patients with UC at the time of serum sampling. No correlation was found between disease activity and presence of ANCA (table 1).

However, when p-ANCA titers were considered, active UC patients had significantly higher titers than active CD patients ($p < 0.01$). There was also a significant difference in p-ANCA titer between active and inactive UC patients ($p < 0.002$), but not between those with active and inactive CD (figure 1). In the group of patients with CD disease activity score was not related to the presence or titer of ANCA. Also, in the group of CD patients with active disease only no relation was observed between activity score and ANCA. In the group of patients with UC endoscopic grading of the disease activity was not related to the presence or titer of ANCA.

The antigenic specificity of p-ANCA was tested by ELISA for five different antigens. We found ANCA with specificity for proteinase 3, myeloperoxidase and/or lactoferrin in 10 out of 67 patients with UC (table 2). In 3 out of 35 patients with CD antibodies to lactoferrin were detected (table 2).

Table II. Specificity of p-ANCA as tested by ELISA for five different antigens. Given are the numbers of sera with positive results.

Antibodies to	Crohn's disease	ulcerative colitis
proteinase-3	0/35	3/67
elastase	0/35	0/67
cathepsin G	0/35	0/67
myeloperoxidase	0/35	3/67
lactoferrin	3/35	3/67
lactoferrin and proteinase	0/35	1/67

Discussion

In the present study we have detected ANCA in the sera of 51% of patients with UC in all but one of the cases producing a perinuclear fluorescence pattern (p-ANCA). The prevalence of p-ANCA in our group of UC patients is somewhat lower than that reported in recent studies. In patients with active disease the prevalence of p-ANCA in our series was 63%. Since we tested sera from consecutive patients from a large locoregional hospital, any bias in patient selection seems improbable. Nevertheless, patient selection may explain some of the differences in the prevalence of p-ANCA in UC between several reports (8-11). In particular, referral centers may study patients with more extended or complicated disease. Also, differences in the technique used for the detection of ANCA may have played a role. Based on our longstanding experience in ANCA-testing (5, 7, 18) and the inclusion of positive and negative controls in every test, we consider false negative results in our series unlikely.

In contrast to the rather low prevalence of ANCA in our UC patients, we observed a strikingly high prevalence of ANCA in our patients with CD. Since ANCA were equally present in patients with ileal, ileocolonic and colonic localization of the disease, we consider diagnostic errors with respect to the differential diagnosis of UC and CD unlikely in our population of CD patients. As such, the presence of ANCA appears to be of limited value in the distinction between UC and CD.

The prevalence of ANCA as detected by indirect immunofluorescence certainly is determined by the cut-off point used in titration for adjudging a positive result. The cut-off point in titration in our study was based on the results obtained from normal control sera. Using this cut-off point of 1:32 only 5% of sera from healthy controls showed a positive result. When, however, titers $\geq$ 1:64 were considered as positive, the prevalence of ANCA was 13% both in the total CD population as well as in the subgroup with active disease. These percentages are more in accordance with other reports (8-11) than the prevalence of ANCA of 40% as found in CD using the 1:32 cut-off point. As for UC patients, the 1:64 cut-off point results in a prevalence of 38% for the total group and 58% for the patients with active disease only.

Interestingly, we did find a difference in the quantity of ANCA between UC and CD. Patients with active UC had significantly higher levels of ANCA than patients with active

CD. Also, ANCA-positive patients with active UC had higher titers than those with inactive UC, although no relation could be observed between the activity score as derived from endoscopy and the presence of ANCA. In patients with CD, titers of ANCA did not differ between patients with active and inactive disease. Longitudinal studies, however, should demonstrate wether the development of active disease is preceded or accompanied by increase in ANCA titer as has been observed for anti-proteinase 3 antibodies in Wegener's granulomatosis (18, 19). Nevertheless, the relation between titers of ANCA and disease activity in UC but not in CD suggests that different mechanisms are involved in the production of ANCA in UC compared to CD. From a practical point of view high-titered ANCA in a patient suspected for inflammatory bowel disease may suggest the presence of active UC.
P-ANCA have been observed, until now, in many different diseases (12). The clinical significance of a positive p-ANCA test therefore is restricted. When the antigenic specificity of p-ANCA is known, such as, e.g., for anti-myeloperoxidase antibodies, its diagnostic significance is greatly increased. Testing the sera from our patients with UC and CD for five different antigenic specificities, i.e. proteinase 3, elastase, lactoferrin, myeloperoxidase and cathepsin G, we found only a minority of the sera positive for defined specificities, in particular for proteinase 3, myeloperoxidase and lactoferrin. The presence of ANCA of defined specificity was not associated with a particular clinical pattern (data not shown). This suggests, at first, that different antigenic specificities are involved in those diseases, and, secondly, that as far as ANCA-specificities are concerned, UC and CD cannot be included in the spectrum of vasculitides which are associated with anti-proteinase 3 and anti-myeloperoxidase antibodies. Studies are now in progress in our laboratory to characterize the antigens involved.

In conclusion, we have demonstrated that the presence of p-ANCA is of limited value in the distinction between UC and CD. High-titered ANCA, however, suggests active UC. Defined ANCA-specificities, in particular antibodies to lactoferrin, are present in a minority of IBD sera only.

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CHAPTER 4.2

ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA) IN INFLAMMATORY BOWEL DISEASE: CHARACTERIZATION AND CLINICAL CORRELATES.

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Summary

Anti-neutrophil cytoplasmic antibodies (ANCA) were detected by indirect immunofluorescence in 34 out of 67 patients with ulcerative colitis (UC, 51%) and in 14 out of 35 patients with Crohn's Disease (CD, 40%). All but one ANCA-positive sera produced a perinuclear pattern of fluorescence (p-ANCA) on ethanol fixed neutrophils. On paraformaldehyde fixed neutrophils 76% of p-ANCA positive sera in UC and 50% of p-ANCA positive sera in CD produced cytoplasmic fluorescence, indicating that, indeed, cytoplasmic antigens are recognized by a considerable number of these sera. By Western blot analysis using whole neutrophil extract as a substrate 46% of sera from patients with UC and 32% of sera from patients with CD showed reactivity with either lactoferrin, polypeptides occurring as a doublet of 66/67 kD MW, or polypeptides occurring as a doublet of 63/54 kD MW, respectively. Identical patterns of reactivity have been observed among p-ANCA positive sera from patients with autoimmune liver disease and rheumatoid arthritis. These data suggest that ANCA of restricted specificities are not specific for IBD but are present in diverse conditions characterized by chronic idiopathic inflammation.

Antibodies directed against cytoplasmic components of the neutrophilic granulocyte (ANCA) have extensively been described as markers for systemic vasculitis and (idiopathic) crescentic glomerulonephritis [reviewed in 1 and 2]. By indirect immunofluorescence (IIF) on ethanol fixed granulocytes two types of ANCA can be distinguished: cytoplasmic or c-ANCA, directed in most of the cases against proteinase 3, and strongly associated with Wegener's Granulomatosis and perinuclear or p-ANCA, in many cases directed to myeloperoxidase and as such associated with idiopathic crescentic glomerulonephritis and different forms of necrotizing systemic vasculitis [3-5].
p-ANCA have not only been detected in systemic vasculitides but also in the sera of patients with inflammatory bowel disease (IBD) [6-13]. Within the spectrum of IBD, p-ANCA have been described as a marker for ulcerative colitis (UC) since the prevalence of ANCA in UC is much higher than that in Crohn's Disease (CD). Saxon and coworkers [8] reported a 68% prevalence in UC versus 20% for CD, Rump and coworkers [10] mentioned a prevalence of 59% in UC and 10% in CD, whereas Oudkerk et al. [13] described a prevalence of 79% in UC and 13% in CD. We recently showed that p-ANCA do occur also in a considerable number of patients with CD (40%) [14]. Titers of p-ANCA, however, were higher in patients with UC compared to CD and titers were higher during active disease than at remission in UC but not in CD [14].
The antigens recognized by p-ANCA in IBD are not fully known. It has, however, been reported that ANCA in IBD are not directed against either myeloperoxidase, proteinase 3, or elastase [11]. Mayet and coworkers [15] and Halbwachs et al. [16] reported ANCA in IBD to be directed against cathepsin G, but this has not been confirmed by Duerr and coworkers [11]. Very recently, Peen et al. [17] described the occurrence of antibodies to lactoferrin as detected by Elisa in a considerable number of patients with UC without relating their findings, however, to the presence of ANCA as detected by immunofluorescence.
The aim of the present study was to determine the antigenic specificities of ANCA in IBD. In order to assess the possible cytoplasmic localization of the antigens involved, ANCA were detected by IIF on both ethanol and paraformaldehyde fixed granulocytes. Western Blotting techniques were used to characterize the antigens recognized by ANCA in IBD.

Patients and methods

Patients and sera:

Sera were studied from patients with ulcerative colitis (UC) (n=67) and Crohn's Disease (CD) (n= 35). The diagnosis of UC or CD was based on accepted clinical and endoscopic criteria supported by histopathology [18]. Data about disease activity were collected retrospectively. CD disease activity was scored as active or inactive based on the Bristol simple index [19]. Active disease was defined as a disease index >= 4 [19]. UC disease activity was scored active or inactive on the basis of history, physical examination, laboratory results, endoscopy, radiology, and the overall clinician's opinion [21]. Active disease was subsequently graded by endoscopic findings in which severity of inflammation was graded 0-2, 2 being spontaneously bleeding, 1 bleeding only after contact, and 0

being non bleeding [21]. 17 out of 67 patients with UC and 13 out of 35 patients with CD took immunosuppressive medication (prednisolone, azathioprine, cyclosporin, or a combination) at the time of serum sampling. The other patients took no medication or sulfasalazine or mesalazine only. Characteristics of the patients are shown in table 1.
Control sera included 252 healthy bloodbank donors (137 males, 115 females, mean age 49 year (range 18-65)) and sera of patients with anti-proteinase 3 or anti-myeloperoxidase antibodies as determined by Elisa [3].

Table I. Clinical characteristics of patients with inflammatory bowel disease at the time of serum sampling.

Diagnosis	Ulcerative colitis n=67	Crohn's disease n=35
male/female	40/27	18/17
mean age (range)	45 (16-78)	41 (20-85)
localization of disease		
ileum, n (%)		12 (34%)
colon, n (%)	67 (100%)	16 (45%)
ileum and colon, n (%)		7 (20%)
number of patients with		
active disease, n (%)	19 (28%)	15 (43%)
activity score, median (range)	2 (0-2)	7 (4-11)
number of patients on		
immunosuppressives, n (%)	17 (25%)	13 (37%)

Antibodies:

Monoclonal antibodies directed against myeloperoxidase (14.15, Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands), proteinase 3 (12.8, CLB, Amsterdam, The Netherlands) and elastase (M752, Dakopatts, Copenhagen, Denmark), and polyclonal antibodies directed against lactoferrin (A186, Dakopatts, Copenhagen, Denmark) and cathepsin G (219358, Calbiochem, San Diego, CA) were used.

Indirect Immunofluorescence:

Detection of ANCA by IIF was done as originally described [22,23] with minor modifications [24]. Test or control sera were applied at a dilution of 1:16 in PBS and at two-fold dilutions up to 1:512. Slides were read by two independent observers, not aware of the clinical diagnosis. A positive ANCA test was considered present when the test was still positive at a dilution of 1:32.
In order to evaluate the cytoplasmic nature of the antigens recognized by p-ANCA we tested all sera by IIF on paraformaldehyde fixed cells. Cytocentrifuged granulocytes were fixed in 0.5% phosphate-buffered paraformaldehyde, pH 8.5, for 10' at RT, after which

detection of ANCA was performed as described.
ANA were detected by IIF on ethanol fixed HEp2 cells.

Western Blotting:
The neutrophil suspension (2 x 10^8 cells/ml) was sonicated three times during 20 seconds in 1 M NaCl containing 5 mM phenylmethylsulfonylfluoride (PMSF, Sigma Chemical Co., St. Louis, MO). Membrane fragments were removed by ultracentrifugation (2 hrs, 100.000 g) and an equivalent to 6 x 10^7 cells was applied on 10% polyacrylamide gels under denaturing but non-reducing conditions. After electrophoresis and transfer to nitrocellulose (Schleicher & Schuell, Keene, NH) the blots were blocked and immunodetection was performed as described previously [25].
A cellular extract of HEp2 cells was prepared following exactly the same procedure.

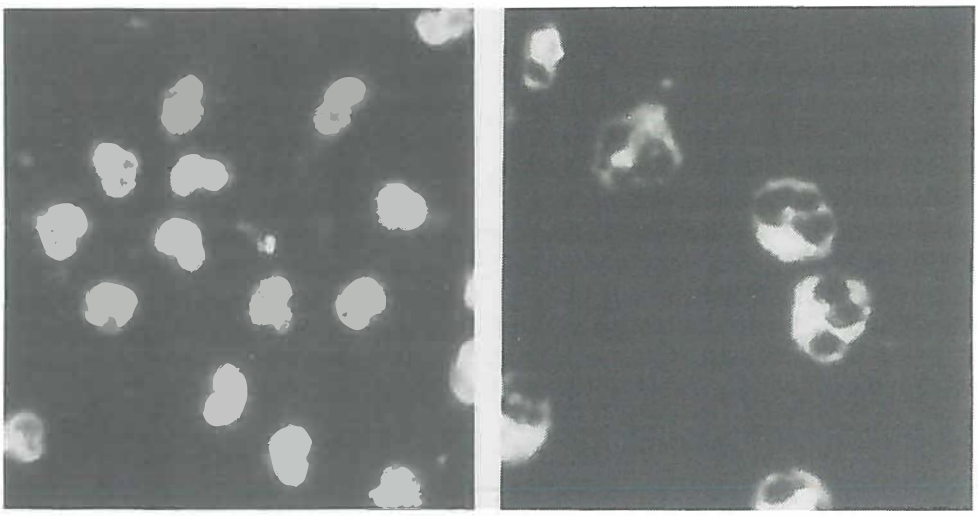
Elisa:
An antigen capture Elisa [4,26] was used to test sera for the presence of either anti-proteinase 3, anti-myeloperoxidase or anti-elastase antibodies. The presence of anti-lactoferrin antibodies was detected by Elisa directly coated with lactoferrin (Serva, Heidelberg, Germany) at a concentration of 7 μg/ml in PBS. Sera were applied at a dilution of 1:50 and diluted twofold till 1:400. Peroxidase conjugated rabbit anti-human Ig, diluted 1:500 was used for detection (Dakopatts P212, Copenhagen, Denmark). Detection of anti-cathepsin G antibodies was performed in a similar way, using cathepsin G (Calbiochem, La Jolla, CA, USA) at a concentration of 1 μg/ml. A test was considered positive when its OD value exceeded the mean + 3 SD of normal controls. These values never exceeded 0.4 OD units (fig 3c).

Statistics:
Statistical analysis of the data was performed using the χ square test. A p-value < 0.05 was considered to represent significance.

Results

Indirect immunofluorescence (IIF):
ANCA were detected by IIF on ethanol fixed granulocytes in 34 out of 67 UC sera (51%), and in 14 out of 35 CD sera (40%). All fluorescence patterns observed were p-ANCA (fig 1A), except for one UC serum which contained c-ANCA. Median titer for ANCA in UC amounted to 128, while the median titer for ANCA in CD was 32. Titers ranged from 1:32 up to $\geq$ 1:512. ANCA in CD did not only occur in 8 out of 16 patients with disease manifestations restricted to the colon (50%), but were detected as well in 3 out of 12 patients (25%) with ileal localization of the disease only, and in 3 out of 7 patients (43%) with ileocolonic localization. p-ANCA were detected in 12 of 252 sera from healthy bloodbank donors (5%). Median titer of ANCA in the positive sera was 64 (range 32-512).
To investigate whether ANCA in IBD recognize cytoplasmic antigens, we screened all sera for antinuclear antibodies (ANA) and performed ANCA testing on paraformaldehyde

fixed cells. ANA were present in 8 out of the 67 UC sera (12%), median titer 32, range 32-128, and in 1 out of the 35 CD sera (3%), titer 32. ANA in IBD produced either a homogeneous or a speckled staining pattern of the nucleus. p-ANCA and ANA were simultaneously present in 6 serum samples. ANA were present in 12 of 252 sera from healthy bloodbank donors (5%), median titer 32, range 32-512. ANCA as detected on



a b

Figure 1a. Perinuclear fluorescence pattern on ethanol fixed granulocytes produced by a serum sample from a patient with ulcerative colitis.
1b. Cytoplasmic fluorescence pattern on paraformaldehyde fixed granulocytes, produced by the same sample.

paraformaldehyde fixed neutrophils were present in 29 out of 67 UC sera (43%), and in 8 out of 35 CD sera (23%). The antibodies produced a diffuse cytoplasmic staining of the granulocyte (fig 1B). The presence of ANCA as detected on paraformaldehyde fixed cells

Table II. Relation between ANCA as detected on ethanol fixed neutrophils and ANCA as detected on paraformaldehyde fixed neutrophils in sera from patients with ulcerative colitis and Crohn's disease.

		ANCA ethanol pos.[1]	ANCA ethanol neg.[1]
UC	ANCA paraform. pos.[2]	26[3]	3
	ANCA paraform. neg.[2]	8	30
CD	ANCA paraform. pos.[2]	7	1
	ANCA paraform. neg.[2]	7	20

[1] number of sera positive (pos.) or negative (neg.) for p-ANCA on ethanol fixed neutrophils.
[2] number of sera showing positive (pos.) or negative (neg.) cytoplasmic staining of paraformaldehyde fixed neutrophils.
[3] one of these sera produced a c-ANCA pattern on ethanol fixed neutrophils.

was correlated with the presence of p-ANCA as detected on ethanol fixed neutrophils ($p < 0.01$), both for UC and CD (table 2). However, 8 sera from UC patients that produced p-ANCA on ethanol fixed neutrophils did not produce cytoplasmic fluorescence on paraformaldehyde fixed cells. Three of these 8 sera were positive for ANA in a homogeneous pattern. In CD patients we found 7 p-ANCA positive sera to be negative on paraformaldehyde fixed neutrophils. One of these 7 sera was positive for ANA. Otherwise, 3 UC sera and one CD serum produced cytoplasmic staining fluorescence on paraformaldehyde fixed cells, while negative for p-ANCA on ethanol fixed cells.

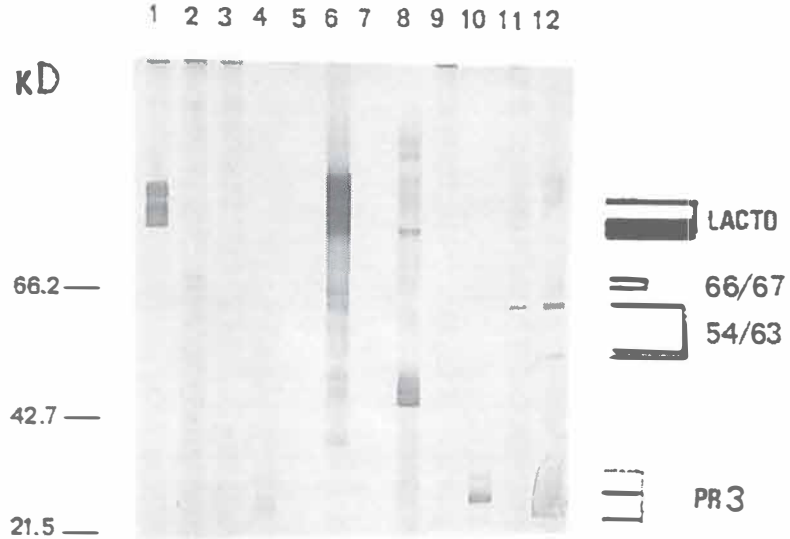


Figure 2. Western blot using sonicated neutrophils as antigenic substrate. Lane 1 serum with anti-lactoferrin antibodies, lane 2 serum with α-67/66 kD antibodies, lane 3, 5, and 7 negative control sera, lane 4 polyclonal anti-cathepsin G antibodies, lane 6 polyclonal anti-lactoferrin antibodies, lane 8 serum with anti-myeloperoxidase antibodies, lane 9 buffer control, lane 10 monoclonal anti-proteinase 3, lane 11 and 12 sera with α-63/54 kD antibodies.

Western blotting

ANCA in IBD recognized several neutrophil derived proteins by Western blotting (figure 2). Using a whole granulocyte sonicate as protein source, reactivity was observed with a 77 kD polypeptide, a 66/67 kD polypeptide doublet, and a 63/54 kD polypeptide doublet, of which the 54 kD polypeptide was a doublet as well. Out of the 67 UC sera 15 reacted with the 77 kD polypeptide, 9 with the 66/67 kD doublet, and 9 with the 63/54 kD combination. Out of the 35 CD sera 6, 3, and 2 sera reacted with the respective polypeptides. Table 3 shows the frequency of reactivity with either of these bands in relation to the presence of ANCA as detected by IIF on ethanol fixed and on paraformaldehyde fixed neutrophils, both for the UC and the CD sera.

To assess the potential of ANCA of known specificity to recognize their target antigens on neutrophil protein blots used in the present study, we tested ANCA controls of known specificity. They showed reactivity with proteins of the expected size, i.e. a 29 kD triplet for anti-proteinase 3, a 110 kD polypeptide together with fragments of 55, 42 and 14 kD for anti-myeloperoxidase, and polypeptides of 30 kD for anti-elastase and anti-

Table III. Reactivity of sera from patients with inflammatory bowel disease with neutrophil proteins as detected by Western blotting.
Relation to the presence of ANCA as detected by indirect immunofluorescence on ethanol fixed and paraformaldehyde fixed neutrophils, respectively.

	Ulcerative colitis			Crohn's disease		
	all sera	pANCA+[2]	paraform +[3]	all sera	pANCA+[2]	paraform +[3]
lactoferrin[1]	15	11	10	6	2	3
n (%)	(22%)	(33%)	(34%)	(17%)	(14%)	(38%)
67/66 kD[1]	9[4]	7[4]	8[4]	3	1	1
n (%)	(13%)	(21%)	(28%)	(9%)	(7%)	(13%)
63/54 kD[1]	10[4]	8[4]	7[4]	2	0	1
n (%)	(13%)	(24%)	(24%)	(6%)	(0%)	(13%)
total[1]	31	24	23	11	3	5
n (%)	(46%)	(73%)	(79%)	(32%)	(21%)	(63%)

[1] number (percentage of sera reacting with specific proteins
[2] sera that produced a perinuclear fluorescence pattern on ethanol fixed granulocytes
[3] sera that produced a cytoplasmic fluorescence pattern on paraformaldehyde fixed granulocytes
[4] one of these sera reacted with lactoferrin as well

cathepsin G antibodies (figure 2, anti-elastase not shown). Polyclonal anti-lactoferrin antibodies reacted strongly with the 77 kD fragment, identical to the respective UC and CD sera, which suggests that the sera recognizing the 77 kD fragment are directed against lactoferrin.
In total we could identify the target proteins in 24 of the 33 p-ANCA positive UC sera and in 3 of the 14 p-ANCA positive CD sera. Furthermore, 8 UC sera negative for ANCA by IIF reacted with either lactoferrin (n=4), the 67/66 kD doublet (n=2) or the 63/54 kD combination (n=2). In CD also, 8 sera negative for ANCA by IIF recognized either lactoferrin (n=4), the 67/66 kD doublet (n=2) or the 63/54 kD combination (n=2). Thirteen out of the 15 sera positive for ANCA on ethanol fixed cells and negative on paraformaldehyde fixed cells did not show reactivity on Western blot. Figure 3 shows the relation between ANCA titer and antigenic specificity. Especially in UC, most of the high titered ANCA could be characterized.
Sera of healthy controls negative for p-ANCA did not show reactivity on Western blot. Out of the 12 p-ANCA positive sera of healthy controls 2 sera showed reactivity with the 67/66 kD doublet and 1 serum with lactoferrin. Using a whole HEp2 cell sonicate as protein source, sera reacting with the various polypeptides from the neutrophil extract showed no reactivity. ANA positive sera of defined specificities showed reactivity with their respective polypeptides on the HEp2 blots (results not shown).

Elisa:

The antigenic specificity of p-ANCA was tested by Elisa for five different antigens. Antibodies to myeloperoxidase were detected in 3 UC sera, of which one was positive for

p-ANCA. Antibodies to proteinase 3 were detected in 3 UC sera, of which one had been scored positive for c-ANCA. The other two sera had been scored positive for p-ANCA. Anti-lactoferrin antibodies were detected in 3 CD patients positive for p-ANCA and in 4 p-ANCA positive UC patients as well. The relation between titers of ANCA by IIF and OD values in Elisa is shown in figure 3C. All sera positive for anti-lactoferrin by Elisa had been scored positive for anti-lactoferrin by Western blotting. Antibodies to elastase or cathepsin G were not detected. In summary, the antigenic specificity of 3 out of 14 pANCA in CD and 9 out of 33 pANCA in UC was established by Elisa. Furthermore, one UC serum positive for c-ANCA appeared to be directed against proteinase 3 by Elisa.

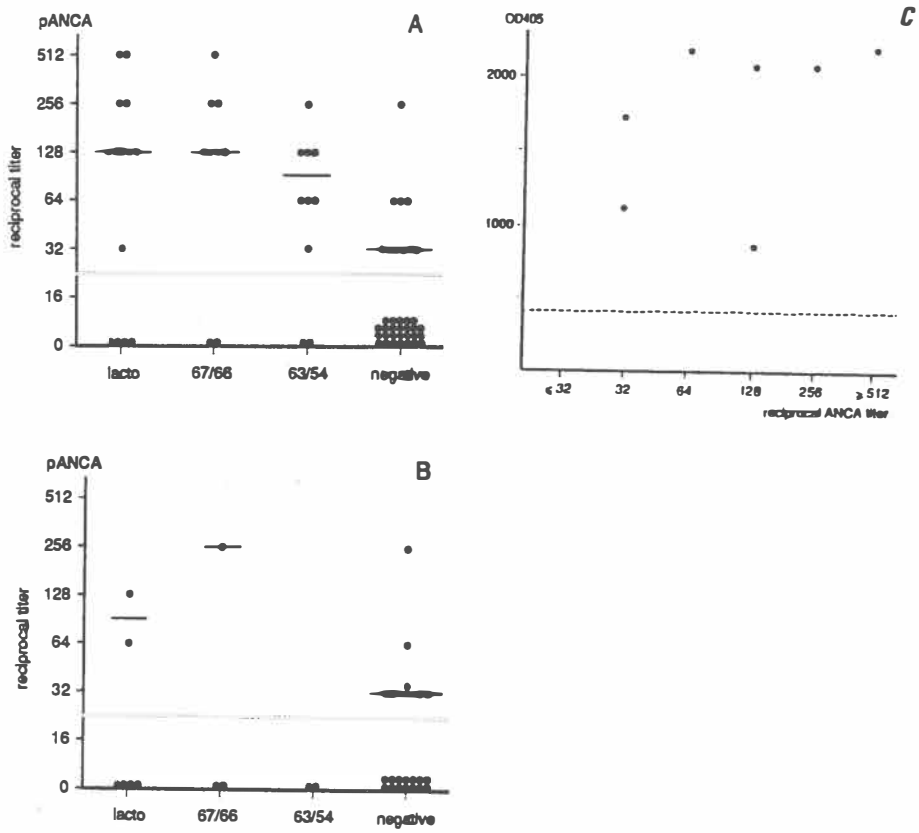


Figure 3. ANCA titers of sera either reacting with lactoferrin, 67/66 kD doublet or 63/54 kD combinations, or being negative on Western blot from patients with ulcerative colitis (A) and Crohn's disease (B). Median titers are represented by solid lines, discrimination between positive and negative is represented by the dashed lines. (C) Relation between ANCA titers and OD values of samples positive for antibodies to lactoferrin by Elisa.

Discussion

Recently, ANCA have been reported in inflammatory bowel disease (IBD). In the present study we detected ANCA in 51 % of patients with UC and 40% of patients with CD. When the analysis was restricted to patients with active disease the prevalence of p-ANCA in our series was 63% (14). The prevalence of p-ANCA in our group of UC patients is somewhat lower than that reported in recent studies. Since we tested sera from consecutive patients form a large locoregional hospital, any bias in patient selection seems improbable. Nevertheless, patient selection may explain some of the differences in the prevalence of p-ANCA in UC between several reports [8-18]. In particular, referral centres may study patients with more extended or complicated disease. Also, differences in the technique used for the detection of ANCA may have played a role. Based on our longstanding experience in ANCA-testing [4,5] and the inclusion of positive and negative controls in every test, we consider false negative results in our series unlikely.

In contrast to the rather low prevalence of ANCA in our UC patients, we observed a strikingly high prevalence of ANCA in our patients with CD. Since ANCA were equally present in patients with ileal, ileocolonic and colonic localization of the disease, we consider diagnostic errors with respect to the differential diagnosis of UC and CD unlikely in our population of CD patients. As such, the presence of ANCA appears to be of limited value in the distinction between UC and CD, although high titered ANCA were more frequently found in patients with active UC [14].

All but one of the ANCA-positive sera produced a perinuclear pattern of fluorescence (p-ANCA) on ethanol fixed neutrophils. As most of these p-ANCA produced a cytoplasmic staining on paraformaldehyde fixed neutrophils, the antigens recognized by the antibodies are apparently mainly localized in the cytoplasm of the neutrophils.

However, 15 sera positive for p-ANCA on ethanol fixed cells were negative on paraformaldehyde fixed neutrophils. Since 4 of these sera were positive for ANA, their presence might have been misinterpreted as ANCA. Otherwise, those p-ANCA may be directed against one or more antigens that are no longer recognized after paraformaldehyde fixation. Western blotting analysis showed that the latter explanation is most probable: 13 of the aforementioned 15 sera did not show reactivity on Western blot. Probably, these sera recognize as yet uncharacterized antigen(s) that are either lost or damaged during paraformaldehyde fixation and are also not recognized on blot due to the denaturing conditions used in the latter technique.

ANCA in IBD are not directed against one specific antigen, but recognize several antigens. On Western blot we found reactivity of IBD sera with either lactoferrin or with as yet uncharacterized proteins, i.e. a 67/66 kD protein doublet or a combination of two proteins of 63 and 54 kD molecular weight. Reactivity was in most cases exclusive for one of these three proteins or protein combinations. Some sera, however, showed reactivity with both lactoferrin and either 67/66 or 64/54 kD polypeptides. The latter two combinations of ANCA antigens, not previously described, have to be further characterized. Reactivity of the sera with the afore mentioned proteins was observed only when using a neutrophil extract and not when using a HEp2 cell extract, indicating that the sera most probably recognize myeloid specific antigens. The 67/66 kD proteins probably constitute a granular protein, as they could be demonstrated in a degranulate of PMNs

(data not shown). In contrast to the dat of Mayet [15] and Halbwachs [16] reactivity of IBD sera with cathepsin G was not observed, although the latter antigen was present in our neutrophil sonicate and was recognized under the conditions used in the immunoblotting technique as demonstrated by using a polyclonal antibody to cathepsin G. Very recently, after this study had been completed, IgG class antibodies to lactoferrin as detected by Elisa were described in 50% of patients with UC and 8% of those with CD [17]. Although their prevalences in the latter study differ somewhat from those observed in our study, possibly due to differences in patient selection and/or disease activity, both studies show that antibodies to lactoferrin occur both in UC and CD.

Using Western blot analysis we could identify the antigenic specificity of almost all of the ANCA positive sera in UC. Reactivity with the above mentioned proteins, however, was observed as well in some IBD sera that were negative for ANCA by IIF. Enhanced sensitivity of the Western blotting technique compared to the immunofluorescence technique might underlie this discrepancy. Furthermore, it is conceivable that the antigens are lost during fixation or are not accessible for their cognate antibodies in the immunofluorescence test. The Western blotting technique proved to be more sensitive than the Elisa as well, since only 7 out of 15 anti-lactoferrin positive sera were detected by Elisa. In sera from CD patients, however, most of the ANCA could not be characterized suggesting that different antigens are, possibly, involved in this disease. This could be in agreement with the results of ANCA testing on paraformaldehyde fixed cells as 7 out of 14 p-ANCA positive CD sera did not show reactivity on neutrophils fixed with the latter fixative. ANCA in IBD can thus be divided into ANCA that produce cytoplasmic fluorescence on paraformaldehyde fixed cells and recognize either lactoferrin or uncharacterized proteins of 67/66 and 63/54 kD, and ANCA that do not stain paraformaldehyde fixed cells. These latter ANCA are mainly found in CD and probably recognize as yet unknown antigen(s).

ANCA recognizing either lactoferrin, the 67/66 kD or the 63/54 kD polypeptides have also been observed in rheumatoid arthritis, primary sclerosing cholangitis, autoimmune chronic active hepatitis and primary biliary cirrhosis [26-28]. In contrast to the specificity of anti-proteinase 3/c-ANCA for Wegener's granulomatosis, p-ANCA directed to either lactoferrin, the 67/66 kD doublet or the 63/54 kD combination are not specific for one disease entity, but might in fact be a marker of chronic inflammation on an autoimmune background.

The pathogenetic role for ANCA in inflammatory bowel disease is still unclear. Since patients with active UC have significantly higher levels of ANCA than patients with active CD and ANCA positive patients with active UC have higher titers than those with inactive UC [12], levels of ANCA may change, at least in UC, in relation to disease activity. Longitudinal studies, however, are required to investigate whether the development of active disease is preceded or accompanied by increase in ANCA titer as has been observed for anti-proteinase 3 antibodies in Wegener's granulomatosis [4,23]. In addition, studies on ANCA and disease duration in IBD should be performed as we found ANCA in autoimmune liver disease and in rheumatoid arthritis to be related with longstanding disease [25, 27]. Whether antibodies to lactoferrin and other neutrophil proteins in ulcerative colitis and Crohn's disease are just an epiphenomenon of inflammation or maintain the inflammatory process by inducing both degranulation and respiratory burst in primed neutrophils [29], remains to be established. Preliminary results show that IgG fractions of sera with antibodies to lactoferrin indeed induce the respiratory burst and

degranulation of primed neutrophils, strongly suggesting that ANCA in IBD are able to maintain or even amplify the inflammatory process.

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CHAPTER 5

ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA) IN JUVENILE CHRONIC ARTHRITIS.

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Summary

In order to evaluate the diagnostic significance of anti-neutrophil cytoplasmic antibodies in childhood we assessed the prevalence of ANCA in juvenile chronic arthritis of either oligoarticular, polyarticular, or systemic onset. In addition, the prevalence of ANCA was investigated in other diseases of childhood characterized by chronic inflammation on autoimmune background, such as cystic fibrosis, juvenile diabetes mellitus, and connective tissue diseases. ANCA were detected in the sera from 35% of JCA patients (n=93), and in only 7% of patients with other inflammatory diseases (n=61). Regarding the onset type of JCA, ANCA were present in 44% of patients with JCA of oligoarticular onset, in 36% of patients with polyarticular onset, and in 16% of patients with JCA of systemic onset.
The presence of ANCA was not related to the presence of rheumatoid factor, prolonged disease duration, or more progressive disease. However, ANCA were less frequently detected in samples drawn during disease remission than in those from active disease, and titers tended to be higher during active disease. All but one of the ANCA positive sera produced a peri-nuclear fluorescence pattern on ethanol fixed granulocytes. However, on neutrophils fixed with paraformaldehyde either a cytoplasmic (14%) or a nuclear (23%) staining pattern was observed, suggesting both cytoplasmic and nuclear autoantibodies occur in JCA.
Further characterization studies showed that ANCA in JCA are not directed against proteinase 3, elastase, or myeloperoxidase. On Western blots ANCA in JCA incindentally showed reactivity with either lactoferrin (5%) or two polypeptides of 66/67 kD (9%). In conclusion, both ANCA and granulocyte-specific ANA occur in JCA and are of limited clinical value.

Anti-neutrophil cytoplasmic antibodies (ANCA) have been described as sensitive and specific markers for systemic vasculitis and (idiopathic) crescentic glomerulonephritis (reviewed in 1 and 2). At least two types of ANCA can be distinguished by indirect immunofluorescence on ethanol fixed neutrophils. One type shows a characteristic cytoplasmic fluorescence pattern (c-ANCA), which, in most of the cases, is produced by antibodies to proteinase 3 (3). The presence of those antibodies is strongly associated with Wegener's Granulomatosis (4). The second type of ANCA demonstrates a (peri)nuclear fluorescence pattern on ethanol fixed neutrophils. This pattern is, in many cases, produced by antibodies to myeloperoxidase (5-7). Antibodies to myeloperoxidase, but not the mere presence of p-ANCA, are associated with crescentic glomerulonephritis and other forms of vasculitis (5-8). p-ANCA have also been detected in sera from patients with ulcerative colitis and Crohn's disease, in patients with autoimmune liver diseases such as primary sclerosing cholangitis and autoimmune chronic active hepatitis, and in rheumatoid arthritis (RA) (9-17). The antigens recognized by ANCA in those conditions have not been definitely characterized. In RA, however, antibodies to lactoferrin occur in some 13 % of the patients (16,17). P-ANCA have furthermore been detected in sera of patients with diverse conditions such as Sweet's syndrome and HIV infections (18-20). The perinuclear fluorescence pattern of ANCA in many of the aforementioned conditions has been shown to be an artefact of ethanol fixation. When a cross-linking fixative as paraformaldehyde is used, many p-ANCA positive sera show a cytoplasmic staining pattern suggesting that the antigens involved are cytoplasmic in nature (21).

Juvenile chronic arthritis (JCA) is a severe chronic disorder of childhood, with varying clinical and serological manifestations. There are 3 major subtypes of JCA which are distinguished by clinical characteristics that are evident at onset or during the first 6 months of the disease (22): a. JCA with polyarticular onset in which $\geq$ 5 joints are affected and which occurs in 40% of the patients; b. JCA with oligoarticular onset in which $\leq$ 4 joints are affected occurring in 50% of the patients; and c. the group of patients with systemic onset of JCA, characterized by spiking fever without known cause for at least two weeks, constitutional symptoms and rash at onset. Oligoarticular JCA can be subdivided in type 1, characterized by early onset, a female preponderance, and the frequent occurrence of chronic uveitis, and type 2, with a relatively late onset, a male preponderance, and an association with spondylarthropathies. Prognosis for the systemic onset JCA is poor as well as for polyarticular onset JCA with seropositivity for rheumatoid factor. This latter type resembles the clinical picture of adult rheumatoid arthritis (RA).

The most common reported autoantibodies in JCA are antinuclear antibodies (ANA) and rheumatoid factors (RF). ANA have been reported to occur in between 30-70% of JCA patients (23). ANA are present in the highest frequency in young girls with oligoarticular onset JCA and in older girls with classic adult RA. ANA are rarely detected in systemic JCA, nor in type 2 oligoarticular JCA. As in RA, most of the ANA in JCA are directed against histones. In general, about 10% of the children with JCA are positive for RF.

Autoantibodies that produce a nuclear fluorescence pattern preferentially on ethanol fixed granulocytes were described in 1959 by Calabresi et al. (24). By testing serum samples on different substrates, it was demonstrated that the antibodies were reactive with granulocytes only, or reacted with granulocytes at an at least four-fold higher dilution than with other cells (25). Based on these findings, the antibodies were designated as "granulocyte-

specific antinuclear antibodies" (GS-ANA) (25). Nässberger et al. (26) reported the occurrence of granulocyte-specific anti-nuclear antibodies (GS-ANA) in JCA. Since the above mentioned definition of GS-ANA excludes the simultaneous occurrence of GS-ANA and ANA with comparable titers in one serum sample, the prevalence of GS-ANA might be underestimated. In addition, the relation between GS-ANA, i.e. autoantibodies directed against nuclear constituents, and ANCA, i.e. autoantibodies to cytoplasmic constituents, has not been studied.
This study was undertaken to determine the prevalence of ANCA and GS-ANA in juvenile chronic arthritis, and to elucidate the antigen(s) involved. We related the presence of the autoantibodies to the onset type of JCA, the disease activity, and the duration of disease. We found that ANCA/GS-ANA are a marker for JCA, but lack specificity for a particular onset type of JCA. ANCA in JCA are generally not directed against defined ANCA antigens such as myeloperoxidase, proteinase 3, elastase, lactoferrin or cathepsin G.

Materials and Methods.

Patients and controls

Juvenile chronic arthritis: The study included 93 patients with juvenile chronic arthritis (JCA). All patients met the criteria of the European League against Rheumatism for JCA (22). Each patient was included in the cross-sectional part of the study in which we used the first serum sample available. From several patients more than one serum sample was available. Forty-one patients had oligoarticular onset JCA, 33 patients had polyarticular onset, and 19 had systemic onset of JCA. Characteristics of the patients are given in table I. From 18 patients we were able to select both a serum sample during active disease and a sample taken during inactive disease. Activity of the arthritis was scored on formal joint assessments: swelling and/or tenderness, pain, and limitation of motion.
Disease controls: The study included serum samples of 30 patients with cystic fibrosis, 10 patients with juvenile diabetes mellitus, 6 patients with systemic lupus erythematosus, 10 patients with juvenile onset of mixed connective tissue disease, and 5 patients with juvenile dermatomyositis. Sera were drawn from consecutive patients seen at the out-patient clinic.

Healthy controls: Sera from 30 normal children were analyzed as well. Furthermore, sera from 252 healthy bloodbank donors were included. Characteristics of controls are given in table 2.

Antibodies of defined specificity:

Mouse monoclonal antibodies (MAbs) directed against myeloperoxidase (14.15 Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB), Amsterdam, The Netherlands), proteinase 3 (12.8, CLB) and elastase (M752, Dakopatts, Copenhagen, Denmark), and polyclonal antibodies directed against lactoferrin (A186, Dakopatts) and cathepsin G (219358, Calbiochem, La Jolla, CA) were used.
Sera from patients with ANCA of defined specificity: anti-myeloperoxidase, anti-proteinase 3, anti-elastase, anti-lactoferrin and anti-cathepsin G, were included. In addition,

reference sera were included from patients with ANA of defined specificities: anti-double stranded DNA, anti-ENA, and anti-histone.

Table I. Demographic and clinical characteristics of 93 JCA patients grouped according to onset type of disease.

onset type	oligoarticular	polyarticular	systemic
number of patients (male/female)	41 19/22	33 12/21	19 7/12
mean age at onset (years) (range)	5.5 (1-15)	8.0 (0-18)	5.3 (1-14)
mean duration of disease (years) (range)	3.0 (0-10)	3.5 (0-18)	0 (1-7)
positive for rheumatoid factor (n)	1	7	0
positive for ANA (n)	23	17	1
HLA-B27 positive (n)	11	5	0
uveitis (n)	3	2	0
use ofimmunosuppressive drugs at the time of testing (n)	3	6	12
C-reactive protein at the time of testing (mg/L, range ±SD)	9.7 ±3.7	18.1 ±8.3	45.2 ±17.2

Table II. Demographic characteristics of disease controls and healthy controls

	n=	m/f	mean age (years)	mean duration of disease (years)
Juvenile disease controls				
cystic fibrosis	30	15/15	11.4	
mixed connective tissue disease	10	3/7	18.6	11.3
diabetes mellitus	10	6/4	13.4	6.1
systemic lupus erythematosus	6	1/5	11.5	2.3
dermatomyositis	5	1/4	8.4	2.2
Healthy controls				
children	30	14/16	7.7	
healthy adults	252	137/115	49	

Indirect Immunofluorescence:
Testing for ANCA was done according to Wiik (27) as agreed on the first international workshop on ANCA with minor modifications (28). 1:20 to 1:640 serial dilutions in PBS of patient or control sera were tested. Slides were read by two independent observers not aware of the clinical diagnosis. A titer of $\geq$ 1:40 was considered positive. Fluorescence patterns were classified as classical or c-ANCA, perinuclear or p-ANCA, and atypical, i.e. positive fluorescence different from c-ANCA or p-ANCA. Testing for antinuclear antibodies was performed by indirect immunofluorescence on ethanol fixed Hep2 cells.
To study whether the antigens recognized by p-ANCA were artificially redistributed during ethanol fixation, phosphate buffered paraformaldehyde (0.5%) at pH 8.5 (10 minutes, RT) (12) was used to fix the granulocytes, after which detection of ANCA was performed as described above. In order to evaluate the influence of paraformaldehyde fixation on the localization of nuclear antigens under the conditions described above, we also applied the latter fixative for ANA testing.

Elisa:
An antigen capture Elisa (6) was used to test sera for the presence of either anti-proteinase 3 (anti-PR3), anti-myeloperoxidase (anti-MPO) or anti-elastase (anti-HLE) antibodies. The presence of anti-lactoferrin or anti-cathepsin G antibodies was detected by Elisa directly coated with lactoferrin (LF) (Serva, Heidelberg, Germany), at a concentration of 5 μg/ml in PBS, or with cathepsin G (CG) (Calbiochem), at a concentration of 1 μg/ml in 0.1 M bicarbonate buffer (pH 9.6). Results were considered positive when the value obtained exceeded the mean of 25 normal control sera by more than 3 SD.

Western Blotting:
SDS-PAGE was performed using the method of Laemli. Neutrophils were isolated from normal donor blood by density centrifugation, dextran sedimentation and hypotonic lysis. The neutrophil suspension (2.10^8 cells/ml) was sonificated for three times during 20 seconds in 1 M NaCl containing 5 mM phenylmethylsulfonylfluoride (PMSF, Sigma Chemical Co., St. Louis, MO). Membrane fragments were removed by ultracentrifugation and an extract equivalent to 6.10^7 cells was applied on 10% polyacrylamide gels under denaturing but non-reducing conditions. Subsequently Western blotting was performed according to standard procedures. Serum samples were applied at a dilution of 1:50. ANCA of defined specificity (MPO, PR3), mono- or polyclonal antibodies against MPO, PR3, HLE, LF, and CG, and serum samples of healthy blood bank donors were used as controls.

Statistics:
Statistical analysis was performed using the χ square test and the Wilcoxon rank test.

Detection of ANCA by indirect immunofluorescence

Sera of 93 JCA patients were screened for the presence of ANCA on ethanol fixed granulocytes. ANCA were detected in sera from 33 patients (35%). The fluorescence pattern was invariably perinuclear, except for one serum which contained an atypical ANCA pattern. As is shown in table 3, ANCA were not equally distributed among the three onset types of JCA: prevalence of ANCA in sera from patients with systemic onset of JCA was much lower compared to that of the oligoarticular and polyarticular types of onset ($p < 0.05$). The number of patients using immunosuppressive drugs was, however, significantly higher in the systemic onset type of JCA compared to the other onset groups ($p < 0.001$). Median titer of ANCA for the three groups was 160 (table 3).

pANCA were detected in 7% of the disease control sera (table 4). Clinical diagnoses of ANCA positive sera were cystic fibrosis (n=2) and mixed connective tissue disease (n=2). Disease controls did not differ from normal controls in which p-ANCA were detected in 5% of the sera (table 4).

ANA were detected in sera from 15 JCA patients (16%) (table 3), 7 with oligoarticular onset JCA (17%), and 8 with polyarticular onset JCA (24%). The ANA patterns observed most frequently were homogeneous and speckled. Median titer was 40 for the oligoarticular onset JCA (range 40-320), and 80 for the polyarticular onset type (range 40-320). Antibodies fullfilling the definition for GS-ANA were detected in 16 sera from oligoarticular onset JCA (39%), in 8 of the polyarticular onset JCA sera (24%) and in 3 of the systemic onset JCA sera (16%) (table 3).

To study whether the antigens recognized by ANCA in JCA were artificially redistributed during ethanol fixation, all sera were screened by IIF on paraformaldehyde fixed neutrophils as well. Interestingly, in JCA of oligoarticular onset 9 of the 18 ANCA positive serum samples were negative on paraformaldehyde fixed cells, while 7 samples produced a nuclear and only two a cytoplasmic staining pattern. Out of the 12 ANCA positive serum samples in polyarticular onset JCA, 7 sera produced nuclear fluorescence on paraformaldehyde fixed neutrophils, 3 sera were negative, and two produced a cytoplasmic fluorescence pattern. 46 out of the 60 ANCA negative samples were negative on the paraformaldehyde fixed cells as well. The remaining 14 samples produced either a cytoplasmic staining pattern (n=8) or a nuclear staining pattern (n=6) (table 5). The nuclear fluorescence pattern observed on the paraformaldehyde fixed cells was correlated with the presence of ANA as detected on HEp2 cells ($p < 0.001$). This was particulary true for patients with ANA positive polyarticular JCA and patients with ANA positive oligoarticular onset JCA who developed polyarticular JCA.

These data show that in fact the definition of ANCA, being autoantibodies to cytoplasmic constituents, should be more elaborated to ensure that ANCA are not confused with other granulocyte-reactive antibodies as GS-ANA or ANA which reacts to nuclear constituents. Assuming that "real p-ANCA" should produce a (peri)nuclear fluorescence pattern on ethanol fixed neutrophils in combination with a cytoplasmic or negative reaction pattern on paraformaldehyde fixed neutrophils, those p-ANCA were detected in 11 out of the 41 oligoarticular onset JCA sera (27%), in 5 out of the 33 polyarticular onset JCA sera (15%) and in 2 out of 19 of the systemic onset JCA sera (11%). In line with the definition

Table III. Prevalence of ANCA and ANA in sera of 93 JCA patients grouped according to onset type of disease.

onset type (n)	oligoarticular (41)	polyarticular (33)	systemic (19)
positive for ANCA n, (%)	18 (44%)	12 (36%)	3 (16%)
median titer (range)	160 (40-≥640)	160 (40-≥640)	160 (40-320)
positive for ANA n, (%)	7 (17%)	8 (24%)	0 (0%)
median titer (range)	40 (40-320)	80 (40-320)	
fullfilling criteria for GS-ANA n, (%)	16 (39%)	8 (24%)	3 (16%)

Table IV. Prevalence of ANCA and ANA in sera from disease controls and healthy controls.

	ANCA positive	median titer (range)	ANA positive	median titer (range)	GS-ANA n, (%)
Disease controls					
cystic fibrosis (n=30)	2 (7%)	40 (40)	0		2 (7%)
mixed connective tissue disease (n=10)	2 (20%)	160 (80-320)	7 (70%)	≥ 640 (40-≥640)	1 (10%)
diabetes mellitus (n=10)	0		3 (30%)	320 (40-≥640)	0
systemic lupus erythematosus (n=6)	0		4 (67%)	≥ 640 (40-≥640)	0
dermatomyositis (n=5)	0		2 (40%)	480 (320-640)	0
Healthy controls					
children (n=30)	2 (7%)	60 (40-80)	2 (7%)	60 (40-80)	2 (7%)
adults (n=252)	12 (5%)	80 (40-640)	12 (5%)	40 (40-≥640)	10 (4%)

of "real p-ANCA" sera producing a perinuclear fluorescence pattern on ethanol fixed neutrophils and a nuclear fluorescence pattern on paraformaldehyde fixed neutrophils as well should be considered as ANA of which some may be GS-ANA. In the JCA population studied here, we detected these ANA in 7 out of the 41 oligoarticular onset JCA sera (17%), in 7 out of the 33 polyarticular onset JCA sera (21%), and in 1 of the 19 systemic onset JCA sera (5%). Of these 5, 3 and 1 sera, respectively, fullfilled the definition of GS-ANA (table 5).

Table V. Detection of ANCA on paraformaldehyde fixed neutrophils in sera of 93 JCA patients grouped according to onset type of disease.

	ANCA as detected on ethanol fixed neutrophils	ANCA as detected on paraformaldehyde fixed neutrophils		
		cytoplasmic pattern	nuclear pattern	negative
oligoarticular	ANCA positive	2 (0)*	7 (5)[4]	9 (0)
	ANCA negative	4 (0)	3 (0)	16 (2)
polyarticular	ANCA positive	2 (1)	7 (3)[3]	3 (0)
	ANCA negative	1 (0)	2 (1)	18 (3)
systemic	ANCA positive	1 (0)	1 (0)[1]	1 (0)
	ANCA negative	3 (0)	1 (0)	12 (0)

*) the number of sera simultaneously positive for ANA are given in brackets, the number of sera fullfilling the definition for GS-ANA in combination with a nuclear fluorescence pattern on paraformaldehyde fixed cells is given in square brackets.

Detection by Elisa and Western Blotting:

All sera were analyzed by Elisa for antibodies to defined neutrophil cytoplasmic antigens, i.e. proteinase 3, myeloperoxidase, elastase, lactoferrin and cathepsin G. Antibodies to myeloperoxidase were found in two JCA patients (2%): one ANCA-IIF positive patient with JCA of oligoarticular onset and one ANCA-IIF positive patient with JCA of polyarticular onset. Antibodies to lactoferrin were found in 4 JCA patients (4%): two ANCA negative patients with JCA of oligoarticular onset, and in two ANCA positive patients with JCA of polyarticular onset. Antibodies to cathepsin G were detected in one ANCA negative patient with JCA of polyarticular onset.

Antibodies to lactoferrin were detected in two of the disease control sera (one with cystic fibrosis, one with mixed connective tissue disease). Antibodies to myeloperoxidase were detected in one of the disease control sera, a case of diabetes mellitus. Antibodies to the above mentioned proteins were not found in any of the sera of the healthy controls.

By Western blotting using sonified neutrophils as antigenic source mono- and/or polyclonal antibodies to myeloperoxidase, proteinase 3, elastase, lactoferrin, and cathepsin G all showed reactivity with proteins of the expected molecular weight (figure 1). Lactoferrin was recognized in Western blot by all sera positive for antibodies to lactoferrin by

Elisa, and, in addition, by one serum of a patient with JCA of oligoarticular onset, that was negative for ANCA by IIF. The serum reacting by Elisa with cathepsin G was also found to react with cathepsin G in Western blot. One of the sera reacting by Elisa with myeloperoxidase did recognize this protein in Western blot, the other one did not. Furthermore, Western blotting revealed reactivity of 8 sera (9%) with a protein doublet of 67/66 kD. Two sera from patients with systemic onset JCA (both of them ANCA negative by IIF), four sera from patients with oligoarticular onset JCA (3 ANCA positive, 1 ANCA negative), and two sera from patients with polyarticular JCA (1 ANCA positive, 1 ANCA negative) recognized this doublet. No correlation could be observed between a specific Western blot pattern and the presence of ANA or nuclear fluorescence on paraformaldehyde fixed cells.

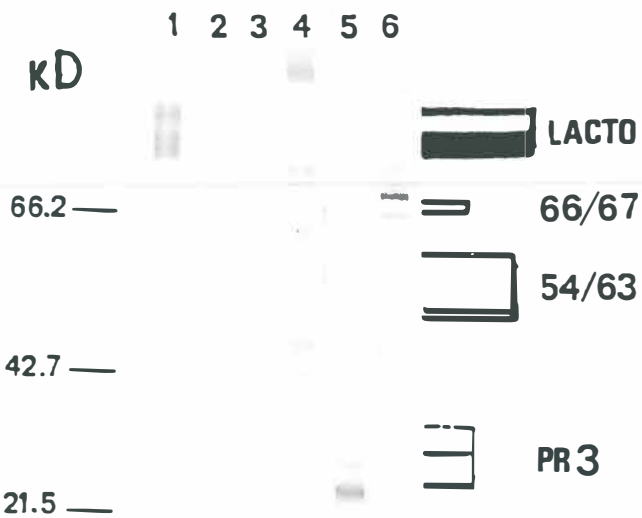

Figure 1. Western blotting with sonicated neutrophils as antigenic substrate; lane 1 serum with anti-lactoferrin antibodies; lane 2 negative contol serum; lane 3 buffer control; lane 4 myeloperoxidase polyclonal antibody; lane 5 proteinase 3 monoclonal antibody; lane 6 serum with 67/66 kD antibodies.

Clinical associations:

As stated, ANCA were present in 44% of patients with oligoarticular JCA, in 36% of those with polyarticular JCA, and in 16% of patients with systemic JCA. Neither the prevalence of ANCA nor the ANCA titer were related to the age at the time of onset, duration of disease, presence of HLA B27 phenotype, or disease activity. However, in patients with inactive disease ANCA were detected significantly less frequently: 72 out of

165 serum samples drawn during active disease were ANCA positive compared to 16 out of 70 serum samples drawn during remission of disease ($p < 0.005$).
From 18 patients paired serum samples were available drawn during active disease as well as during remission. The prevalence of ANCA in active serum samples did not differ from that in the paired inactive serum samples. Median ANCA titer was higher in the ANCA positive samples from active disease (160, range 160-640) in comparison with titers in the remission samples (80, range 40-320) although the difference did not reach statistic significance.

Discussion

The presence of various autoantibodies can be associated with specific onset types of JCA. IgM rheumatoid factor is associated with late onset of polyarticular disease and poor prognosis, while anti-nuclear antibodies have been associated with pauciarticular disease and uveitis. Therefore, we studied the prevalence of antineutrophil cytoplasmic antibodies in JCA and their clinical significance.
ANCA were detected in sera from 35% of the JCA patients studied. Regarding the onset type of JCA ANCA were present in 44% of patients with JCA of oligoarticular onset, in 36% of patients with JCA of polyarticular onset, and in 16% of patients with JCA of systemic onset.
Nässberger et al. (26) reported a prevalence of GS-ANA in JCA of 17%. The highest frequency was observed in polyarticular JCA (35%), followed by oligoarticular JCA (6%). In their study GS-ANA were not detected in systemic onset JCA. However, one should realize that GS-ANA are defined as antibodies that are reactive with granulocytes only or as antibodies that react with granulocytes at an at least four-fold higher dilution than with other cells (25). Since this definition excludes the simultaneous occurrence of GS-ANA and ANA with comparable titers in one serum sample, the prevalence of the former autoantibodies might be underestimated. If we apply the classical definition of GS-ANA on our data, a prevalence of GS-ANA in JCA of 26% was found, which, indeed, is lower than the prevalence of ANCA in JCA. Compared with the data of Nässberger (26) we found a different distribution of GS-ANA among the three JCA onset types: GS-ANA in our population were detected most frequently in patients with oligoarticular onset JCA (39%), followed by polyarticular onset JCA (24%) and systemic onset JCA (16%).
The presence of ANCA seems not to be restricted to one specific onset type, although a far lower prevalence was found in systemic onset JCA. The lower prevalence of ANCA in systemic onset JCA has to be considered with the notion that patients in this subgroup of JCA received significantly more immunosuppressive drugs, a fact that may have influenced our data. However, in the 7 patients with systemic onset JCA who did not receive immunosuppressive drugs, ANCA were not detected, suggesting that the prevalence of ANCA in systemic onset JCA indeed is low. Considering the two types of oligoarticular JCA we found no difference in the prevalence of ANCA. Furthermore, the presence of ANCA was not related to the presence of rheumatoid factor, prolonged disease duration, or more progressive disease, so ANCA cannot be used to make clinical distinctions between the onset types of JCA and their prognosis. ANCA might, however,

be of additional value as a marker for JCA, since ANCA were present in only 9% of disease control sera and in 5% of healthy control sera.
ANCA were less frequently detected in serum samples drawn during remission of the disease suggesting a relation with disease activity. However, studying paired serum samples from 18 patients the prevalence of ANCA was not higher during active disease than during remission. Titers of ANCA, however, tended to be higher during active disease. Longitudinal studies are needed to confirm a possible relation between titers of ANCA and disease activity.
To confirm the cytoplasmic nature of the antigens recognized by ANCA in JCA all sera were screened for ANCA on paraformaldehyde fixed neutrophils as well. Since paraformaldehyde is a crosslinking fixative it prevents the electrostatically induced redistribution of cationic granule proteins such as myeloperoxidase, elastase, and lactoferrin as observed on ethanol fixed cells (21). Surprisingly, we found that only a minority of the ANCA positive sera showed cytoplasmic fluorescence on paraformaldehyde fixed neutrophils, whereas the majority showed nuclear fluorescence on this substrate as well. This is in contrast with ANCA in other chronic inflammatory diseases as rheumatoid arthritis, ulcerative colitis, and autoimmune chronic active hepatitis in which we observed a clear correlation between the presence of (peri)nuclear ANCA on ethanol fixed neutrophils and a cytoplasmic staining pattern on paraformaldehyde fixed cells (12, 13, 17). Obviously, the antigens recognized by ANCA in JCA are different from those recognized by ANCA in the previously mentioned diseases. Since we found a significant correlation between the presence of a nuclear fluorescence pattern on paraformaldehyde fixed cells and the presence of ANA as detected on HEP2 cells, our data suggest a true nuclear localization of the antigens involved. This was furthermore confirmed by applying a stronger fixative, i.e. acetone in combination with 9% formaldehyde as described by Pryzwanski et al. (21). Using this fixative the nuclear fluorescence on paraformaldehyde fixed granuloctyes remained nuclear.
When we defined the presence of (p)-ANCA as a (peri)-nuclear fluorescence on ethanol-fixed neutrophils and a cytoplasmic pattern on paraformaldehyde fixed cells, ANCA were detected at a lower prevalence in our JCA population compared to their prevalence based on the presence of (peri)-nuclear staining only: 27% versus 44% in oligoarticular onset JCA, 15% versus 35% in polyarticular onset JCA, and 5% versus 16% in systemic onset JCA. True granulocyte-specific antinuclear antibodies were observed in a minority of the sera as well (10%).
To further elucidate the nature of the antigen(s) recognized by ANCA in JCA characterization studies were performed. Antigen-specific Elisa's showed that ANCA in JCA do not recognize either proteinase 3, elastase or cathepsin G, antigens that are recognized by ANCA in Wegener's Granulomatosis and systemic vasculitis (3, 29), as well as in Crohn's disease (30). Antibodies to myeloperoxidase, which are associated with idiopathic crescentic glomerulonephritis and different forms of necrotizing systemic vasculitis (7,8) were detected in two sera. These patients had no signs of vasculitis. These findings are consistent with those of Nässberger et al. (26) who reported one out of 96 JCA patients to be positive for anti-myeloperoxidase, and none to be positive for elastase. Antibodies to lactoferrin, which have been detected in about 13% of sera in rheumatoid arthritis, ulcerative colitis, Crohn's disease, and autoimmune liver diseases, were detected in sera from 5 JCA patients (5%).

To further characterize the antigenic specificity of the remaining ANCA positive sera Western blot analysis was performed on all sera. Apart from sera recognizing lactoferrin, we could identify 8 sera (9%) that recognized two polypeptides of 67 and 66 kD. These specificities of ANCA have been detected in rheumatoid arthritis (7%), inflammatory bowel disease (12%), and in autoimmune liver diseases (14%) as well (12, 13, 17). The antigens are not characterized yet. They seem to be myeloid specific since they cannot be demonstrated using HEp2 cells as a substrate, and seem to be granular proteins, as they were demonstrated in a degranulate of PMNs (17).

In conclusion, prevalence and antigenic specificity of ANCA in JCA are clearly different from that in adult onset rheumatoid arthritis or other chronic inflammatory diseases that have been found associated with ANCA. The true cytoplasmic nature of the antigens involved have been ascertained in 14% of the JCA sera studied only, whereas the antigens involved have been characterized only incidentally. Besides, true granulocyte-specific antinuclear antibodies appear to occur in some JCA patients. The clinical significance of ANCA in JCA is restricted although the antibodies occur less frequently in systemic onset JCA and only incidentally in sera from children with different chronic inflammatory disorders. Titers of ANCA tend to fluctuate with disease activity but this should be confirmed in further prospective studies.

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CHAPTER 6.1

ACTIVATION OF GRANULOCYTES BY ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES: A FcγRII-DEPENDENT PROCESS.

A.H. Leontine Mulder, Peter Heeringa, Elisabeth Brouwer, Pieter C. Limburg en Cees G.M. Kallenberg.

Summary

Anti-neutrophil cytoplasmic antibodies (ANCA) have been demonstrated to induce the respiratory burst in primed neutrophils. In this study we have extended the investigations on neutrophil activation by ANCA directed against proteinase 3 (PR3), myeloperoxidase (MPO) and lactoferrin (LF), and we have analyzed the underlying mechanisms. All three ANCA-antigens were expressed on the cell surface of primed neutrophils. Superoxide production assayed both by cytochrome c reduction and oxidation of dihydrorhodamine 123, was induced by heterologous polyclonal anti-MPO and anti-LF antibodies, and ANCA-positive plasma samples. Induction of superoxide production was dose-dependent. $F(ab')_2$ fragments did not induce the respiratory burst. Blockade of Fc-receptors by specific monoclonal antibodies showed that anti-FcγRII antibodies were able to turn off the ANCA-induced respiratory burst, whereas anti-FcγRIII antibodies did not. Plasma samples that induced the respiratory burst did not differ from samples that did not induce superoxide production with respect to ANCA titer, but had higher levels of IgG3 subclass ANCA. The levels of the other subclasses were comparable. We conclude that ANCA-induced activation of primed neutrophils is FcγRII-dependent and appears to be facilitated by antibodies of the IgG3 subclass.

Antibodies directed against cytoplasmic constituents of the neutrophilic granulocyte (ANCA) have extensively been described as markers for systemic vasculitis and (idiopathic) crescentic glomerulonephritis (reviewed in 1 and 2). By indirect immunofluorescence (IIF) on ethanol fixed granulocytes two types of ANCA can be distinguished: cytoplasmic or c-ANCA and perinuclear or p-ANCA. c-ANCA are directed, in most of the cases, against proteinase 3 (PR3), and are strongly associated with Wegener's Granulomatosis (WG). A considerable number of p-ANCA positive sera contain antibodies to myeloperoxidase (MPO) which are associated with idiopathic crescentic glormerulonephritis and different forms of necrotizing systemic vasculitis (3-5). P-ANCA may, however, also be directed against lactoferrin (LF), cathepsin G, or other still unknown antigens, and are detected in various diseases characterized by chronic inflammation such as rheumatoid arthritis, primary sclerosing cholangitis, autoimmune chronic active hepatitis, ulcerative colitis and Crohn's disease (6-11).
A role for ANCA in the pathogenesis of WG has been suggested by the studies of Cohen Tervaert et al. (12, 13). They demonstrated that disease activity of WG is preceded by increase in c-ANCA titer (12), and that early treatment of WG based on changes in c-ANCA levels prevents relapses of the disease (13). The underlying mechanisms remained, however, unclear. Recently, Falk et al. (14) demonstrated that ANCA directed against PR3 or MPO induce, in vitro, the respiratory burst and degranulation of normal donor granulocytes primed with TNFα. Further studies showed that ANCA enhance the adherence of neutrophils to endothelial cells (15) and neutrophil-mediated target cell destruction (16). In addition, as PR3 and MPO also are highly cationic proteins that readily sitck to endothelial cells, ANCA may induce complement-dependent cytotoxicity by binding to their localized antigens (17).
The precise mechanism of ANCA induced neutrophil activation has, however, not been unravelled yet. In the present study we further explored the mechanisms involved in neutrophil activation by ANCA directed to PR3 and MPO as well as ANCA directed to LF which are associated with chronic inflammatory disorders. It proved that granulocyte activation by ANCA of diverse specificities is Fc-dependent and not mediated by $F(ab')_2$ fragments alone. Fc-interaction occurs via the second Fc-receptor (FcγRII). Accordingly, ANCA-induced granulocyte activation is particularly exerted by antibodies of the IgG3 subclass.

Materials and methods

Reagents:

Formyl-methionyl-leucyl-phenylalanine (FMLP, F3506, Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and stored in sterile, pyrogen-free containers at -80 °C. All buffers and media used throughout the purification procedure and the activation experiments were pyrogen free and contained less than 5 pg/ml endotoxin as determined by the Limulus amoebocyte assay.

Sera:

Plasma samples, either obtained from plasmaphoresis material or from freshly drawn

blood, were obtained from patients with either Wegener's Granulomatosis, systemic vasculitis, rheumatoid arthritis, ulcerative colitis or Crohn's disease. All samples were

Table I. Characteristics of plasma samples used in this study.

#	IIF pattern[1]	IIF titer[2]	antigenic specificity[3]	clinical diagnosis[4]
1	c-ANCA	1:128	PR3	WG
2	c-ANCA	1:128	PR3	WG
3	c-ANCA	1:128	PR3	WG
4	p-ANCA	1:128	MPO	SV
5	p-ANCA	1:128	LF	RA
6	p-ANCA	1:512	LF	RA
7	p-ANCA	1:64	LF	RA
8	p-ANCA	1:64	LF	RA
9	p-ANCA	1:128	LF	RA
10	p-ANCA	1:128	LF	UC
11	p-ANCA	1:128	LF	UC
12	p-ANCA	1:128	LF	UC
13	p-ANCA	1:64	LF	UC
14	p-ANCA	1:128	LF	CD
15	p-ANCA	1:64	LF	CD
16	-	-		healthy control
17	-	-		healthy control
18	-	-		RA
19	-	-		RA
20	-	-		SLE

[1] Pattern of fluorescence on ethanol fixed neutrophils; c-ANCA indicating a cytoplasmic pattern and p-ANCA indicating a perinuclear pattern, - = ANCA-negative.
[2] Titer of antibodies as determined by two-fold dilution starting at a dilution of 1:16.
[3] PR3 = proteinase 3, MPO = myeloperoxidase, LF = lactoferrin.
[4] WG = Wegener's Granulomatosis, SV = systemic vasculitis, RA = rheumatoid arthritis, UC = ulcerative colitis, CD = Crohn's disease, SLE = systemic lupus erythematosus.

positive for either c-ANCA or p-ANCA. Characteristics of the samples are given in table I. Control plasma samples consisted of fresh samples from healthy volunteers, samples positive for antinuclear antibodies (ANA) and negative for ANCA derived from patients with rheumatoid arthritis, and a pool of plasma samples from patients with systemic lupus erythematosus (SLE) that contained antibodies to double stranded DNA and were negative for ANCA.
Purified immunoglobulin G fractions were prepared from all of the above mentioned plasma samples by sequential ammonium sulphate precipitation and protein G chromatography (fast flow protein G, Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Antibodies:
Rabbit anti-human myeloperoxidase polyclonal antibodies (A398), rabbit anti-human lactoferrin polyclonal antibodies (A186), and normal rabbit immunoglobulins (X903) were obtained from Dakopatts (Copenhagen, Denmark). Monoclonal antibody IV.3 (anti-

FcγRII, IgG2b) was derived from Medarex Inc. (West-Lebanon, NH), and mAbs CD16 FcR gran 1 (anti-FcγRIII, IgG2a) and 12.8 (anti-PR3, IgG1) were derived from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service (CLB, Amsterdam, The Netherlands). For some experiments $F(ab')_2$ were prepared by digestion with pepsin (P7012, Sigma Chemical Co.) in 0.1 M NaAc pH 3.6 for 16 hrs at 37 °C. To end the reaction the vials were centrifuged for 10 minutes at 13000 g and the supernatant was dialyzed against Hanks balanced salt solution (HBSS, Gibco BRL, Breda, The Netherlands). SDS-PAGE was performed to ensure that the digestion was complete. Fluorescein isothiocyanate (FITC) conjugated $F(ab')_2$ fragments of rabbit immunoglobulins to human IgG (F315, Dakopatts) and FITC conjugated swine immunoglobulins to rabbit immunoglobulins (F205, Dakopatts) were used as secondary antibodies.

Detection of ANCA by IIF:
Detection of ANCA was done as described before (18). Test or control sera were applied in 1:16 to 1:512 serial dilutions. Slides were read by two independent observers, and a titer $\geq$ 1:32 was considered positive.

Characterization of ANCA-specificities by Elisa:
An antigen capture Elisa, as previously described (19), was used to test sera for the presence of antibodies to either PR3, MPO, or elastase. The presence of lactoferrin antibodies was detected by Elisa on plates directly coated with lactoferrin (5 μg/ml, Serva, Heidelberg, Germany). Peroxidase conjugated rabbit anti-human Ig, diluted 1:500, was used for detection (P212, Dakopatts, Copenhagen). Results by Elisa were considered positive when the value obtained exceeded the mean of 30 normal control sera by more than 3 SD.

Isolation of granulocytes:
Peripheral blood from normal volunteers was drawn into vacutainer tubes containing 0.34 M EDTA. The blood was diluted 1:1 in 0.9% NaCl, and polymorphonuclear granulocytes were separated by centrifugation on a Lymphoprep density gradient (Nycomed Pharma AS, Oslo, Norway). Contaminating erythrocytes were removed by hypotonic lysis. Following two washsteps with icecold phosphate buffered saline (PBS), the granulocytes were suspended in HBSS. Prior to the activation experiments the granulocytes were warmed gradually to 37 °C, and treated for 5 minutes with cytochalasin B, 5 μg/ml (18015, Serva, Heidelberg, Germany). Priming of the granulocytes was performed by incubation with recombinant TNFα (rTNFα, Genzyme, Cambridge, MA), 2 ng/ml for 15 minutes.

Surface expression of ANCA antigens:
Surface expression of ANCA antigens was investigated by indirect immunofluorescence, followed by flow cytometric analysis. Freshly isolated granulocytes, either primed or not, were fixed with 1% paraformaldehyde for 10 minutes on ice. Incubation with the primary antibody in a dilution of 1:50 was performed for 45 minutes at 4 °C, followed by incubation with the FITC-conjugated secondary antibody in a dilution of 1:100 for 30 minutes at 4 °C. After each step the cells were extensively washed with and finally resuspended in PBS/BSA. Flow cytometric analysis was performed with 10.10^6 cells/ml

using a FACS STAR (Becton Dickinson, San Jose, CA).

Detection of superoxide production using the ferri-cytochrome C reduction assay:
Superoxide production by granulocytes was determined by measuring the superoxide dismutase (SOD) (S9636, Sigma Chemical Co.) inhibitable reduction of ferri-cytochrome C, discontinously, according to the method of Pick and Mizel (20) with minor modifications. In short, 96-well microtiter plates (F-form, Greiner BV, Alphen a/d Rijn, The Netherlands) were incubated with freshly purified granulocytes (1.10^6 cells/ml), 690 μM ferri-cytochrome C (C7752, Sigma Chemical Co), either 590 U/ml SOD or an equal volume HBSS, and stimulus. IgG preparations were added at a concentration of 80 μg/ml, fMLP was used at a concentration of 0.1 μM. The plates were scanned repeatively at 550 nm using a Titertek multiscan MCC 340 apparatus. Between the readings the plates were kept at 37 °C. Each test was performed in quadruplicate.

Detection of superoxide production using flow cytometry:
Superoxide production by granulocytes was determined additionally by measuring the oxidation of dihydrorhodamine 123 (DHR) (D632, Molecular Probes, Eugene, OR). Freshly isolated cells, either primed or non-primed, (1.10^6 cells/ml) were incubated for 15 minutes with DHR (1 μg/ml) at 37 °C. Next, stimuli were added to the cells followed by an incubation step of 30 minutes. Cells were pelleted at 200 g and resuspended in ice-cold HBSS at a concentration of 10. 10^6 cells/ml. Emission was measured at 530 nm at a FACS STAR apparatus. The concentrations of the stimuli were identical to those used in the cytochrome C reduction assay.

IgG subclass detection of ANCA by Elisa:
IgG subclass detection of ANCA was performed as described previously (21). In brief a crude granule extract was isolated by nitrogen cavitation of neutrophils as described by Borregaard et al (22). Microtiter plates were coated either with the extract at a protein concentration of 20 μg/ml in 0.1 M carbonate buffer, pH 9.6, or with lactoferrin (5 μg/ml) in PBS for 1.5 hour at 37 °C. The plates were incubated with human sera for 1 hour at a dilution of 1:100 and subsequently with subclass specific monoclonal antibodies (anti-human IgG1 clone MH161-1, anti-human IgG2 clone HP6014, anti-human IgG3 clone MH163-1-Mo5, anti-human IgG4 clone MH164-4, CLB, Amsterdam) at a dilution of 1:250. Antibody binding was detected with alkaline phosphatase conjugated goat-anti-mouse IgG (A7157, Sigma Chemical Co.). Values were expressed in OD units after subtraction of blanks.

Results

Expression of ANCA antigens on the cell surface:
The ANCA antigens PR3, MPO, and LF were not present on the surface of neutrophils in freshly drawn blood immediately fixed with paraformaldehyde. A representative example of LF expression is shown in fig 1a. Isolation of neutrophils resulted in low surface expression of the ANCA antigens as shown in fig 1b for the expression of LF. Priming of

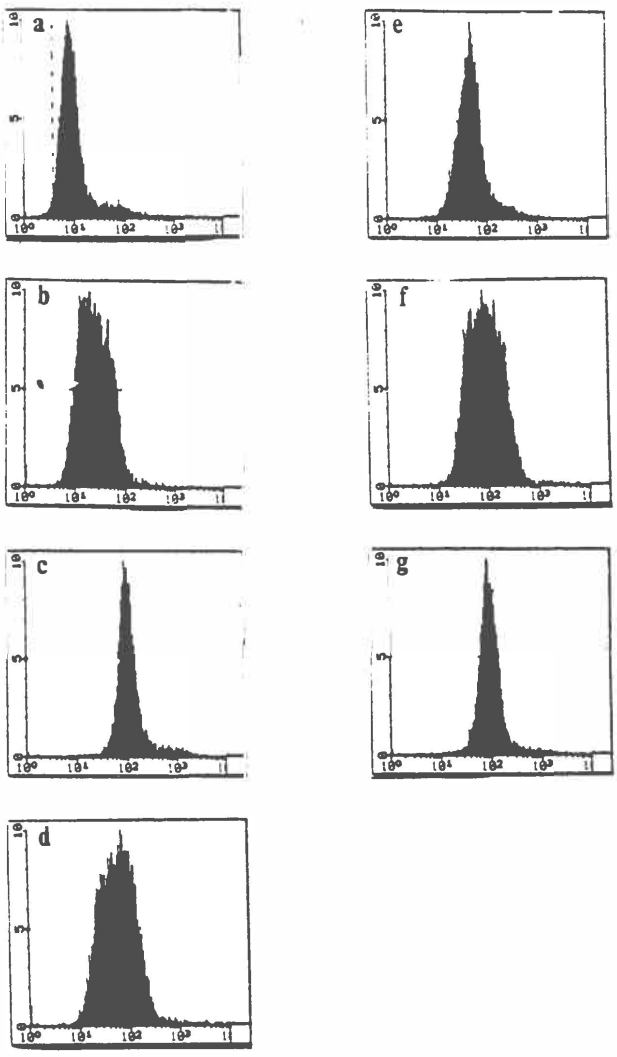

Figure 1.

A. Expression of lactoferrin on the surface of granulocytes in freshly drawn peripheral blood. The dotted line represents non-specific binding.

B. Expression of lactoferrin on the surface of freshly purified granulocytes.

C-E. Expression of lactoferrin (C), myeloperoxidase (D), and proteinase 3 (E) on the surface of rTNFα primed granulocytes as detected by IgG from plasma samples.

F-G. Expression of myeloperoxidase (F) and lactoferrin (G) on the surface of rTNFα primed granulocytes as detected with $F(ab')_2$ preparations from plasma samples positive for MPO-ANCA and LF-ANCA, respectively.

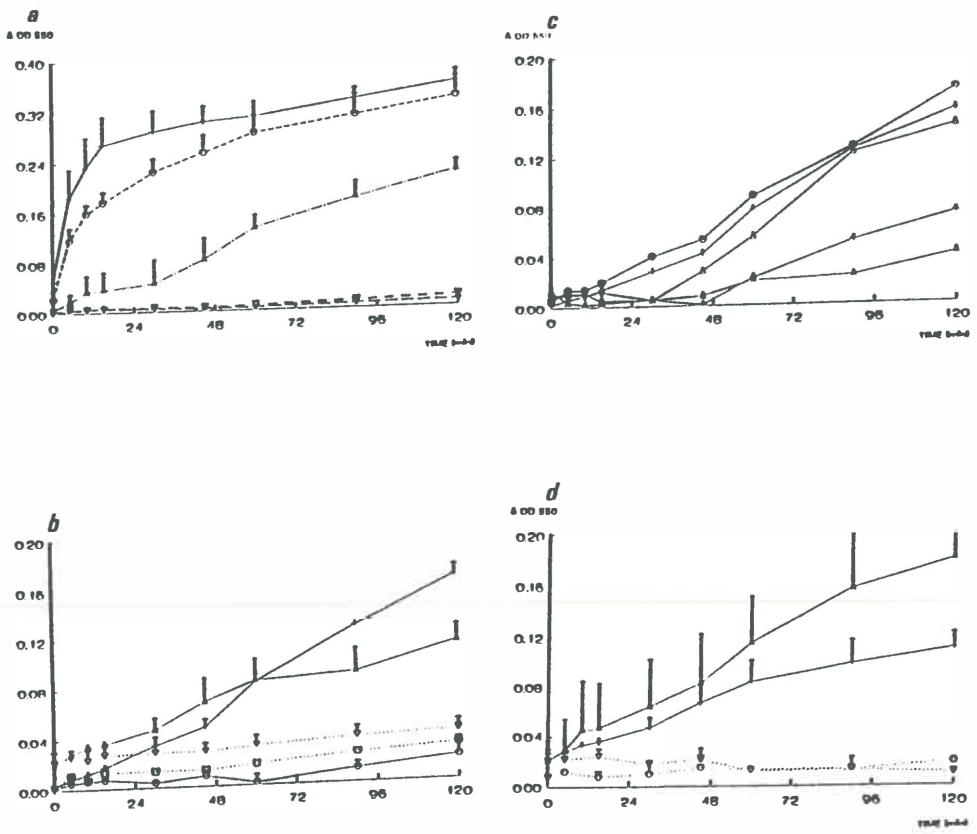

Figure 2. Superoxide production by granulocytes as measured by the superoxide dismutase inhibitable cytochrome c reduction assay. The horizontal axis is the time axis of superoxide production (in minutes), the vertical axis shows the superoxide dismutase inhibitable cytochrome c reduction as expressed as the difference in OD 550 units. Data are presented as the mean + s.d. of three respective experiments.

A. Superoxide production of primed (rTNFα, 2 ng/ml) granulocytes induced by fMLP (+), buffer (◇), heterologous lactoferrin IgG (o), and normal rabbit immunoglobulin (▽). Superoxide production in non-primed cells by heterologous anti-lactoferrin (▲) IgG is shown as well.

B. Superoxide production of primed (solid lines) and non-primed (dotted lines) cells induced by patients IgG directed against MPO (▲, ▽) and LF(+, □) and superoxide production of primed cells by normal IgG (o).

C. Dose dependency of superoxide production of primed granulocytes induced by LF-ANCA in primed granulocytes. Doses shown are 80 (o), 40 (+), 20 (△), 10 (+) and 5 (▲) μg IgG / ml respectively.

D. Superoxide production of primed neutrophils induced by IgG (solid lines) or $F(ab')_2$ fragments (dotted lines) from anti-MPO (+, o) and anti-LF (▲, ▽) positive plasma samples.

neutrophils with 2 ng/ml rTNFα for 15 minutes at 37 °C resulted in significantly increased expression of PR3, MPO, and LF on the cell surface (fig 1c-e). The presence of those ANCA antigens was demonstrated at the cell surface using heterologous polyclonal immunoglobulins to the respective antigens as well as using purified IgG derived from plasma samples positive for PR3-ANCA, MPO-ANCA or LF-ANCA, respectively. Also, $F(ab')_2$ fragments of those IgG fractions recognized the respective antigens on the cell surface. Fig 1f and g demonstrate the surface expression of MPO and LF as detected by $F(ab')_2$ fragments from plasma samples positive for MPO-ANCA and LF-ANCA respectively.

Superoxide production as measured by cytochrome c reduction:
Superoxide production was measured by the superoxide dismutase inhibitable reduction of ferri-cytochrome c. The chemotactic peptide formyl-methionyl-leucyl-phenylalanine (fMLP) which is known to induce the respiratory burst in granulocytes was used as positive control. As shown in figure 2a fMLP induced the superoxide production by purified granulocytes, whereas buffer did not. Heterologous polyclonal anti-lactoferrin or anti-myeloperoxidase antibodies induced the respiratory burst in primed granulocytes. In freshly isolated non-primed granulocytes induction of the respiratory burst did not occur during the first 30 minutes. Normal rabbit immunoglobulin did not induce the respiratory burst in primed neutrophils (fig 2a). Next, we tested IgG fractions from ANCA positive plasma samples (anti-PR3, n=3, anti-MPO, n=1, anti-LF, n=11) for their capacity to induce the respiratory burst in primed neutrophils. All three anti-PR3 positive IgG fractions, the anti-MPO positive fraction, and 2 out of 11 anti-LF positive IgG fractions induced superoxide production in primed but not in non-primed cells (fig 2b). The remaining 9 IgG fractions, all positive for anti-LF, did not induce the respiratory burst, not even when tested at higher concentrations (the concentration was increased stepwise from 80 μg/ml up to 1 mg/ml). The amount of superoxide production induced by the ANCA samples capable to induce neutrophil activation proved to be dose-dependent (fig 2c). However, the capacity to induce the respiratory burst and ANCA titer, either measured by IIF or by antigen-specific ELISA, did not correlate (fig 3).
In order to assess the Fc-dependency of ANCA-induced neutrophil activation $F(ab')_2$ fragments were applied in molar base equal to the IgG preparations. $F(ab')_2$ fragments either from heterologous polyclonal immunoglobulins or derived from patients IgG were not able to induce superoxide production (fig 2d), although they were able to recognize their respective antigens on the surface of primed neutrophils.
IgG derived from patients with anti-nuclear antibodies (samples 18-20) or from normal healthy controls (samples 16-17) did not induce the respiratory burst in primed neutrophils (fig 2b). No relation was observed between the presence of IgG rheumatoid factor in the plasma samples and the capacity of plasma samples to induce the respiratory burst (data not shown).

Superoxide production as measured by the oxidation of dihydrorhodamine 123:
Since the ferri-cytochrome c reduction assay measures the amount of extracellular superoxide, this method may not be sensitive enough to measure low amounts of superoxide production. Therefore, we used the superoxide specific oxidation of dihydrorhodamine as detected by flow cytometry. As shown in figure 4a fMLP induced superoxide

production, whereas buffer did not. Polyclonal anti-lactoferrin and anti-myeloperoxidase antibodies induced the respiratory burst in primed cells, while hardly any production was seen in unprimed cells (fig 4b). $F(ab')_2$ fragments did not induce superoxide production in primed cells (fig 4c). IgG derived from patients with anti-nuclear antibodies or from normal healthy controls did not induce oxidation of dihydrorhodamine (data not showm).

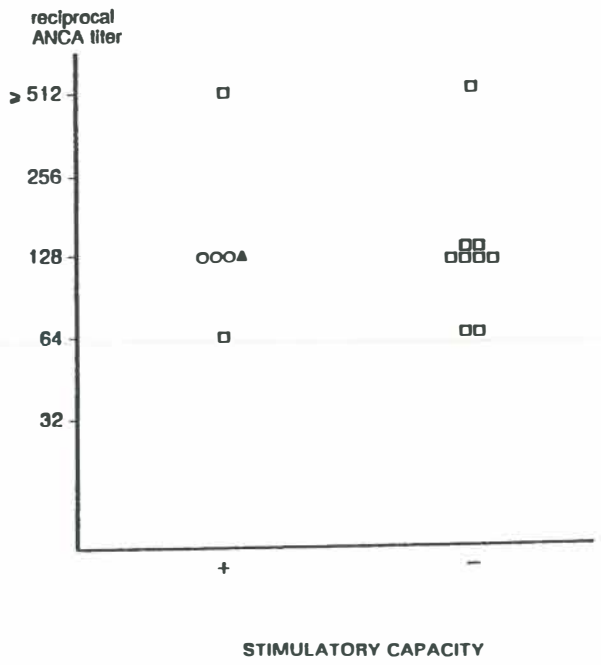

Figure 3. Titer of ANCA as measured by IIF and capacity of IgG to induce superoxide production in plasma samples from patients with ANCA of diverse specificities. □ represents anti-lactoferrin IgG, ▲ represents anti-myeloperoxidase IgG and o represents anti-proteinase 3 IgG.

Blockade of Fcγ-receptors:

Since it appeared that the presence of Fc fragments on the immunoglobulins was required to induce the respiratory burst, blockade of Fcγ-receptors was supposed to prevent the induction of the respiratory burst. As shown in figure 5 $F(ab')_2$ fragments of the monoclonal antibody IV.3 directed against FcRII (10 μg/ml) prevented the respiratory burst in its initial phase as measured by the cytochrome c reduction assay of primed neutrophils

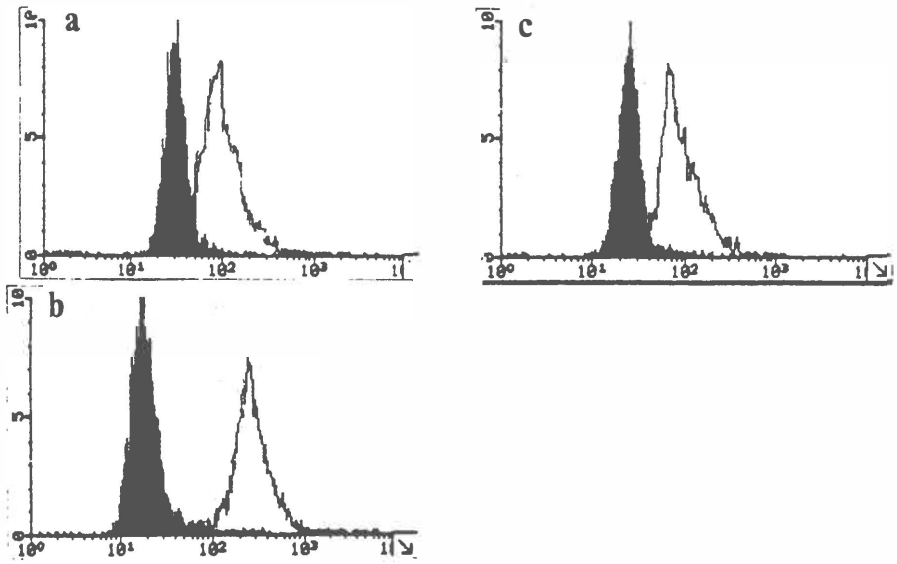

Figure 4. Superoxide production by granulocytes as measured by the oxidation of dihydrorhodamine
A. fMLP induced superoxide production in comparison with buffer
B. Polyclonal anti-lactoferrin induced superoxide production in primed granulocytes in comparison with non-primed cells.
C. Polyclonal anti-lactoferrin induced superoxide production in primed cells by IgG in comparison with $F(ab')_2$ fragments.

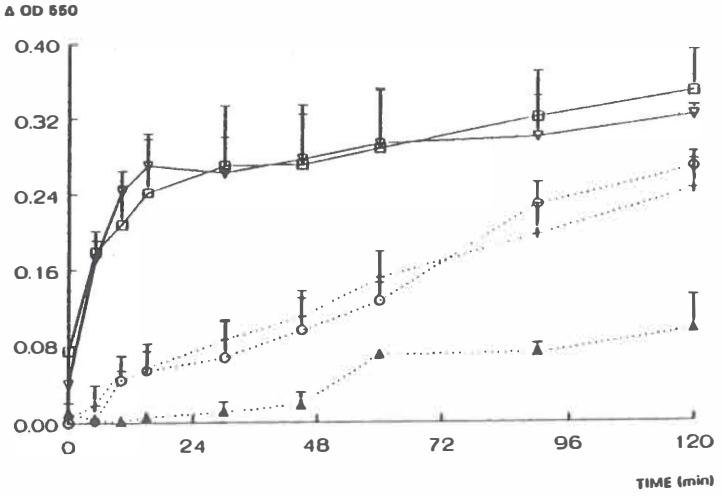

Figure 5. Superoxide production as measured by the superoxide dismutase inhibitable cytochrome c reduction assay:
Induction of the respiratory burst by fMLP in the presence of anti-FcRII (▽) and in the presence of FcRIII (□) and induction of the respiratory burst by anti-lactoferrin (o) either in the presence of anti-FcRII (▲) or anti-FcRIII (+) monoclonal antibodies.

stimulated with polyclonal anti-myeloperoxidase or anti-lactoferrin antibodies. fMLP induced respiratory burst was not inhibited when th anti-FcγRII monoclonal antibody was added. The $F(ab')_2$ fragments of the monoclonal antibody CLB gran1 directed against FcRIII (20 μg/ml) did not influence the cytochrome c reduction assay (fig 5). Using the dihydrorhodamine assay similar results were obtained. Blockade of FcγRII prevented the ANCA induced respiratory burst to occur, whereas blockade of FcγRIII had no effect (table 2). Simultaneous blockade of both receptors had no additional effect (data not shown).

IgG subclass distribution:

Since the various IgG subclasses have different affinities for the Fc -receptors, we studied the IgG subclass distribution in IgG preparations that induced the respiratory burst and in preparations that did not. ANCA titers between both groups were comparable (fig. 3). As shown in fig. 6, ANCA IgG preparations that induced the respiratory burst in primed neutrophils had higher levels of IgG3 than ANCA IgG preparations that did not induce the respiratory burst ($p < 0.005$). Levels of the other IgG subclasses were comparable.

Table II. Fc-dependency of ANCA induced superoxide production as measured by the dihydrorhodamine assay.

	Mean fluorescence intensity		
stimulus[1]	+ buffer	+ Fc RII[2]	+ Fc RIII[2]
fMLP	150	152	142
α-LF	235	122	230
Rb Ig	91	94	92

Given is a representative example of three experiments.
[1] α-LF = heterologous polyclonal anti-lactoferrin antibodies, Rb Ig = normal rabbit immunoglobulins.
[2] addition of $F(ab')_2$ fragments of anti-FcRII (MoAb IV.3) and anti-FcRIII (CLB gran1), respectively.

Discussion

This study confirms and extends data that the intracellularly localized antigens recognized by ANCA become accessible at the cell surface after priming of granulocytes, and that interaction of the antigens with the respective autoantibodies results in induction of the respiratory burst. In addition, we demonstrate that this process is FcRII dependent and may be preferentially achieved by IgG3 subclass antibodies.

Neutrophil activation is thought to contribute significantly to tissue destruction in several diseases, including vasculitis, rheumatoid arthritis, and inflammatory bowel disease. Falk et al. (14) were the first to report that PR3- and MPO-ANCA may play an active role in neutrophil activation. They demonstrated, *in vitro*, that after a "priming" event the ANCA antigens are expressed on the surface of the neutrophil, which makes them available for

interaction with ANCA. Subsequently, this interaction leads to activation of the neutrophil, resulting in the production of oxygen radicals and release of lytic enzymes which may contribute to tissue destruction as seen in ANCA associated diseases. As such, the presence of ANCA might directly be related to neutrophil activation. The mechanisms by which neutrophils are activated by ANCA has not been extensively studied yet. Fujimoto et al. (23) demonstrated that after incubation with ANCA neutrophils show translocation

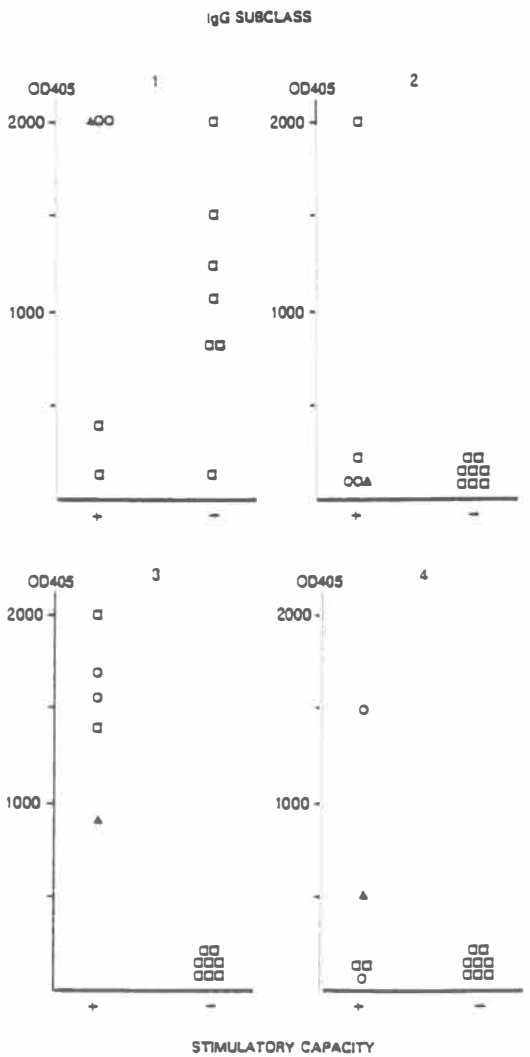

Figure 6. IgG subclass distribution of ANCA IgG as determined by Elisa in relation to their capacity to induce the respiratory burst. On the X-axis the IgG fractions are divided in samples that can induce granulocyte activation (+) and samples that cannot induce granulocyte activation (-). On the Y-axis OD units (after subtraction of blanks) for antigen-specific IgG-subclass ANCA are given. Fig A represents IgG1, B IgG2, C IgG3 and D IgG4 ANCA. □ represents anti-lactoferrin IgG, ▲ represents anti-myeloperoxidase IgG and o represents anti-proteinase 3 IgG.

of protein kinase C to the cell membrane. This translocation is the initial step in the activation of protein kinase C, which participates in the assembly and the activation of the NADH oxidase system.

We tested ANCA of various specificities for their capacity to induce the respiratory burst in primed neutrophils. We observed, in accordance with prior studies (14,24), that the ANCA antigens proteinase 3, myeloperoxidase and lactoferrin are expressed on the surface of rTNFα primed neutrophils, whereas these antigens were hardly expressed on freshly purified neutrophils. Incubation of primed granulocytes with ANCA of defined specificities, either heterologous or derived from plasma samples from ANCA positive patients, resulted in induction of the respiratory burst. The largest amount of superoxide production was induced by heterologous anti-lactoferrin antibodies, probably related to the higher expression of lactoferrin on the cell surface in comparison to myeloperoxidase and proteinase 3. ANCA induced superoxide production was dose-dependent, and other laboratories (24) have shown that ANCA induced superoxide production is antigen-specific. It is not known at this moment if other antibodies that recognize surface antigens are able to stimulate primed neutrophils as well.

It has been suggested that neutrophil activation by ANCA results from $F(ab')_2$ dependent binding of surface antigens only (14). Using the cytochrome c reduction assay, we could, however, not detect induction of the respiratory burst by $F(ab')_2$ fragments from ANCA of either specificity, although those $F(ab')_2$ preparations still recognized the ANCA antigens of the cell surface of primed granulocytes. To control for a possibly low sensitivity of the cytochrome C reduction assay, superoxide production was also detected by the oxidation of dihydrorhodamine as measured by flow cytometry. This system might be more sensitive as it measures the intracellular superoxide production. Using this system the $F(ab')_2$ preparations from ANCA positive IgG fractions again failed to induce the respiratory burst in primed granulocytes. In their initial report Falk et al. (14) mentioned only one $F(ab')_2$ preparation from an anti-MPO positive serum that induced the respiratory burst as tested by the chemiluminescence assay. We tested IgG- as well as $F(ab')_2$ preparations from ANCA positive plasma samples for their capacity to induce the respiratory burst using the chemiluminescence assay in which either a fluorimeter or a scintillation counter was used for detection. Again, $F(ab')_2$ fragments failed to induce the respiratory burst in primed neutrophils whereas IgG fractions did (data not shown). Recently, Keogan et al. (24) found 5 out of 6 $F(ab')_2$ ANCA preparations to produce neutrophil activation as demonstrated by a chemiluminescence assay. They could, however, not inhibit this response with superoxide dismutase but did inhibit their reaction with azide and salicylhydroxamic acid. This demonstrates that their assay measures not superoxide production but the active myeloperoxidase system.

From the aforementioned data we conclude that ANCA induced activation of primed neutrophils is dependent on the Fc fragments of the IgG molecule. Along these lines we hypothesize that interaction of the ANCA antigens of the neutrophil surface with the autoantibodies leads to an in situ immune complex formation which activates neutrophils by way of their Fcγ-receptors. Since rTNFα primed neutrophils mainly express Fcγ-RIII and Fcγ-RII, we next investigated the effect of blockade of these receptors on ANCA induced neutrophil activation was investigated. We used $F(ab')_2$ fragments of monoclonal antibodies against these receptors, which have been proven to block the receptor without inducing activation (25). Indeed, we found that the induction of the respiratory burst by

ANCA could be prevented by blockade of Fcγ-RII, while blockade of Fcγ-RIII had no effect. Blockade of both receptors had no additional effect, and the fMLP induced respiratory burst was not affected by the inhibitory monoclonal antibodies. Thus, ANCA induced neutrophil activation is dependent of the Fcγ-RII receptor. This conclusion is in accordance with the studies of Reibmann et al. (26) and Huizinga et al. (25) who showed that FcγRII is the most important receptor in eliciting the respiratory burst while Fcγ-RIII is involved more preferentially in binding of immunecomplexes and is unable by itself to transduce transmembrane signals. Other studies, however, have shown that both receptors are evenly important for superoxide production (27, 28), or suggest that FcγRIII might even be the main pathway in immunecomplex activation of granulocytes (29, 30).

During our study we observed that not all of the ANCA positive sera were able to induce the respiratory burst. Although activation of granulocytes was dependent on the concentration of ANCA in a particular sample, ANCA titers in samples capable of stimulation were comparable to those that were incapable of stimulation. Out of the 11 sera positive for anti-lactoferrin only 2 IgG fractions were found to be able to induce the respiratory burst, despite the fact that in general anti-lactoferrin antibodies gave rise to the strongest stimulation. The distribution of IgG subclasses of ANCA in plasma samples that stimulated primed granulocytes, however, appeared to be different from that in plasma samples that did not stimulate. Stimulating samples contained high concentrations of IgG3-ANCA, whereas non-stimulating samples did not. Since IgG3 binds strongly to FcγRII, this IgG subclass may preferentially be involved in ANCA activation. In this regard it is interesting that Huizinga et al. (31) observed that human neutrophils bind IgG3 complexes approximately three times faster than IgG1 complexes, whereas IgG2 and IgG4 complexes are not bound. IgG3 complexes were bound both by FcγRII and FcγRIII. The IgG subclass distribution of ANCA in Wegener's Granulomatosis has been studied by Brouwer et al (21). They found that ANCA in WG are predominantly of the IgG1 and IgG4 class, but the additional presence of IgG3 was associated with the development of renal involvement, one of the most serious sequelae of the disease. Further longitudinal studies, however, have to be performed to investigate the relationship between IgG subclasses of ANCA, neutrophil activation and disease activity of the ANCA associated diseases.

In conclusion, IgG from ANCA positive samples with specificity for PR3, MPO, and LF are capable of inducing the respiratory burst in primed neutrophils, although not all ANCA-positive samples do stimulate neutrophils. The activation process is dependent of the expression of the ANCA antigens on the cell surfacte and the presence of FcγRII receptors. Preliminary data suggest that the IgG3 subclass, in particular, is involved in the induction of the respiratory burst.

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CHAPTER 6.2

ACTIVATION OF GRANULOCYTES BY ANTI-NEUTROPHIL CYTOPLASMIC ANTIBODIES (ANCA) IN WEGENER'S GRANULOMATOSIS: A PREDOMINANT ROLE FOR THE IgG3 SUBCLASS OF ANCA.

AHL Mulder, CA Stegeman, CGM Kallenberg.

Summary

To study ANCA induced granulocyte activation in relation to disease activity in Wegener's granulomatosis (WG) paired serum samples from patients with WG from active and inactive disease were analyzed for their capacity to activate primed normal donor granulocytes. The capacity of serum IgG fractions to induce the respiratory burst was significantly correlated with the ANCA titer ($p < 0.005$). Furthermore, ANCA positive IgG fractionss from active disease sera better induced the respiratory burst than ANCA positive IgG fractions from inactive disease sera, even when comparing sera with identical titer.

In order to explore why IgG fractions from active disease better induced the respiratory burst than IgG from inactive diseaase we assessed changes in IgG subclass distribution of ANCA during active disease and remission. Changes in the capacity to activate granulocytes were significantly related to changes in the relative amount of IgG3 subclass of ANCA ($p < 0.001$), and not to changes in the relative amount of IgG1 or IgG4 subclass of ANCA. These data suggest that the increase in neutrophil activating capacity of ANCA from inactive to active disease is, at least in part, based on the relative increase of the IgG3 subclass of ANCA that occurs during active disease.

Anti-neutrophil cytoplasmic (auto)antibodies (ANCA) directed against the third serine proteinase of human granulocytes, proteinase 3 (PR3), have been described as sensitive and specific markers for Wegener's granulomatosis (WG) (1-3). These autoantibodies produce a cytoplasmic staining pattern (c-ANCA) of ethanol fixed neutrophils in an indirect immunofluorescence assay. A role for c-ANCA in the pathogenesis of WG has been suggested by various longitudinal studies demonstrating that disease activity of WG is preceded by increase in c-ANCA titer (3, 4) and that early treatment based on changes in c-ANCA levels prevents relapses of the disease (5). The underlying mechanisms have, however, not been elucidated.

Recently, Falk et al (6) demonstrated that ANCA directed against PR3 and myeloperoxidase (MPO) induce, in vitro, the respiratory burst and degranulation of normal donor granulocytes primed with TNFα. These data were confirmed by other laboratories (7, 8). Further studies showed that ANCA enhance the adherence of neutrophils to endothelial cells and neutrophil-mediated target cell destruction (9, 10). In addition, as PR3 and MPO are cationic proteins that readily stick to endothelial cells or even may be expressed by these cells (11), ANCA may induce complement-dependent cytotoxicity by binding to their localized antigens (12).

The precise mechanism of ANCA induced neutrophil activation has not been unravelled yet. We and others demonstrated that ANCA induced neutrophil activation is dependent on Fc-receptors on the neutrophils (8, 13), in particular the FcγRII. In addition, our data suggested a predominant role for ANCA of the IgG3 subclass in neutrophil activation since sera capable of inducing the respiratory burst had increased levels of antigen-specific IgG3 antibodies compared to sera that did not induce granulocyte activation (8). The latter findings were based on a transsectional study in a few patients.

In the present study we evaluated whether active disease in WG is associated not only with an increase in ANCA titer but also with changes in functional characteristics of ANCA. We analyzed paired serum samples from ANCA positive patients drawn at the time of active and inactive disease, respectively, for their capacity to induce the neutrophil respiratory burst. In addition, we determined whether the capacity of ANCA-positive sera to activate primed neutrophils was related to disease activity. Furthermore, we related neutrophil activating capacity to IgG subclass distribution of ANCA. The results show that IgG isolated from patients sera during active disease better induced the respiratory burst than IgG from ssera drawn during remission, and that changes in levels of IgG3 ANCA correlate with changes in activating capacity of the antibodies.

Materials and methods

Sera:

Serum samples were obtained from 17 patients with biopsy proven Wegener's granulomatosis (WG) at the moment of diagnosis and during clinical remission withing a period of 3 to 12 months after the time of diagnosis. The diagnosis of WG was established according to clinical and histological criteria (14) and all patients fulfilled the criteria for the classification of WG as described by the American College of Rheumatologists (15). Disease activity of WG was scored using a disease activity index and a distinction between

major and minor disease activity was made as described before (5, 16). Briefly, minor disease activity was defined as active lesions of WG in the upper- or lower airways without evident vasculitic activity in other organs. Major disease activity was defined by renal involvement with deteriorating renal function with red blood cell casts or biopsy proven necrotizing glomerulonephritis, pulmonary involvement with impending respiratory failure, cerebral vasculitis, or acute abdomen or massive gastro-intestinal haemorrhage due to vasculitis.
All samples were positive for c-ANCA. Characteristics of the patients are given in table I. Control plasma samples consisted of freshly drawn samples from healthy volunteers (n=4), or samples positive for antinuclear antibodies (ANA) (n=2).
Purified immunoglobulin G fractions were prepared from all of the above mentioned plasma samples by sequential ammonium sulphate precipitation and protein G chromatography (fast flow protein G, Pharmacia Fine Chemicals AB, Uppsala, Sweden).

Table I. Characteristics of the patients

men/women	12/5
mean age at diagnosis[1]	47.1 (range = 20-72)
limited WG/ generalized WG	7/10
disease activity during diagnosis[2]	19.2 (range = 8-35)
disease activity during remission[2]	0.76 (range = 0-4)

[1] given in years
[2] scored as reported in ref. 16

Reagents:

Formyl-methionyl-leucyl-phenylalanine (FMLP, F3506, Sigma Chemical Co., St. Louis, MO) was dissolved in DMSO and stored in sterile, pyrogen-free containers at -80 °C. All buffers and media used throughout the purification procedure and the activation experiments were pyrogen free and contained less than 5 pg/ml endotoxin as determined by the Limulus amoebocyte assay.

Detection of ANCA by IIF:

Detection of ANCA was performed as described before (1). Test or control sera were applied in 1:16 to 1:512 serial dilutions. Slides were read by two independent observers, and a titer $\geq$ 1:32 was considered positive.

Characterization of ANCA-specificities by Elisa:

An antigen capture Elisa, as previously described (17), was used to test sera for the presence of antibodies to either PR3, MPO, or elastase. Results by Elisa were considered positive when the value obtained exceeded the mean of 30 normal control sera by more than 3 SD.

Isolation of granulocytes:
Peripheral blood from normal volunteers was drawn into vacutainer tubes containing 0.34 M EDTA. The blood was diluted 1:1 in 0.9% NaCl, and polymorphonuclear granulocytes were separated by centrifugation on a Lymphoprep density gradient (Nycomed Pharma AS, Oslo, Norway). Contaminating erythrocytes were removed by hypotonic lysis. Following two washsteps with icecold phosphate buffered saline (PBS), the granulocytes were suspended in Hanks Balanced Salt Solution (HBSS). Prior to the activation experiments the granulocytes were warmed gradually to 37 °C, and treated for 5 minutes with cytochalasin B, 5 μg/ml (18015, Serva, Heidelberg, Germany). Priming of the granulocytes was performed by incubation with recombinant TNFα (rTNFα, Genzyme, Cambridge, MA), 2 ng/ml for 15 minutes.

Detection of superoxide production using the ferri-cytochrome C reduction assay:
Superoxide production by granulocytes was determined by measuring the superoxide dismutase (SOD) (S9636, Sigma Chemical Co.) inhibitable reduction of ferri-cytochrome C, discontinuously, according to the method of Pick and Mizel (18) with minor modifications. In short, 96-well microtiter plates (F-form, Greiner BV, Alphen a/d Rijn, The Netherlands) were incubated with freshly purified granulocytes (1.10^6 cells/ml), 690 μM ferri-cytochrome C (C7752, Sigma Chemical Co), either 590 U/ml SOD or an equal volume HBSS, and stimulus. IgG preparations were added at a concentration of 80 μg/ml, fMLP was used at a concentration of 0.1 μM. The plates were scanned repeatively at 550 nm using a Titertek multiscan MCC 340 apparatus. Between the readings the plates were kept at 37 °C. Each test was performed in quadruplicate. The activation of the neutrophils was expressed as the difference in OD 550 nm between the ferri-cytochrome c reduction in the absence and in the presence of superoxide dismutase; this ΔOD is directly proportional to the amount of superoxide produced.

IgG subclass detection of ANCA by Elisa:
IgG subclass detection of ANCA was performed as described previously (19). In brief, a crude granule extract was isolated by nitrogen cavitation of neutrophils as described by Borregaard et al (20). Microtiter plates were coated with the extract at a protein concentration of 20 μg/ml in 0.1 M carbonate buffer, pH 9.6 for 1.5 hour at 37 °C. The plates were incubated with human sera for 1 hour at a dilution of 1:100 and subsequently with subclass specific monoclonal antibodies (anti-human IgG1 clone MH161-1, anti-human IgG3 clone MH163-1-Mo5, anti-human IgG4 clone MH164-4, CLB, Amsterdam) at a dilution of 1:250. Antibody binding was detected with alkaline phosphatase conjugated goat-anti-mouse IgG (A7157, Sigma Chemical Co.). Values were expressed in OD units after subtraction of blanks.

Statistical analysis:
Statistical analysis was performed using the Mann-Whitney rank sum test, Pearson product-moment correlation coefficient, and the Spearman rank correlation coefficient.

Neutrophil activation and ANCA titer

Paired samples from 17 patients with WG drawn at the moment of diagnosis and subsequently 3-12 months after diagnosis were assayed for their capacity to induce the neutrophil respiratory burst using the superoxide dismutase inhibitable reduction of ferricytochrome c. As is shown in figure 1, all but one of the samples were able to induce the neutrophil respiratory burst in primed normal donor granulocytes, while normal control sera or sera containing anti-nuclear antibodies did not. The mean ΔOD550 nm as a measure for the amount of superoxide production, induced by ANCA-positive IgG fractions was 0.078 ± 0.049 compared to 0.007 ± 0.003 induced by control IgG fractions ($p < 0.01$). The capacity to induce the respiratory burst was significantly related to the titer of ANCA as determined in the corresponding IgG fraction ($r = 0.535$, $p < 0.005$, $n=34$).

Comparing sera from the moment of active disease and remission, respectively, (figure 1) we found that IgG purified from sera during active disease showed significantly higher induction of the respiratory burst than IgG purified from sera taken during disease remission ($p < 0.005$). The median ANCA titer at the moment of active disease was higher than at the moment of remission (256 (range 32-512) vs 64 (range 32-512)) although the difference was not statistically significant. When, however, sera drawn at the moment of active disease were compared with sera having an identical ANCA titer but drawn during inactive disease, it proved that sera from the moment of active disease induced a higher level of respiratory burst than sera from inactive disease (fig 2). Comparing paired serum samples from individual patients obtained on the moments of active disease and remission, respectively, we found again that active disease samples induced a higher degree of granulocyte activation than sera from disease remission. The PMN-activating capacity of ANCA was not related to renal impairment, and occurred in patients with generalized WG as well as in patients with limited WG (fig 1).

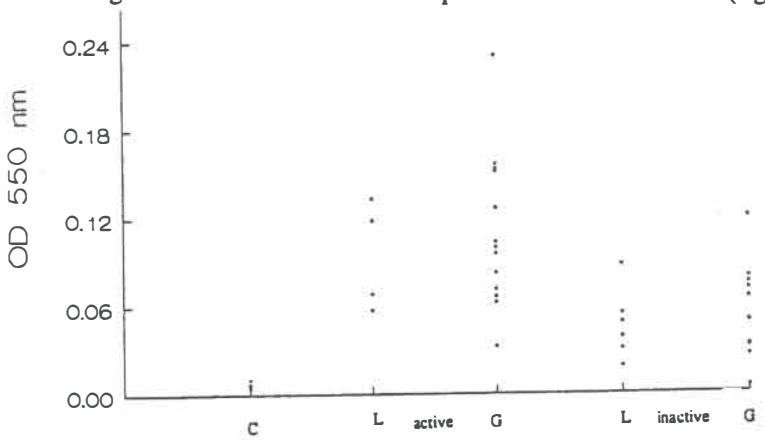

Figure 1. ΔOD 550 nm as a measure for the amount of superoxide production of normal PMNs induced by IgG fractions derived from sera from either normal controls (C), active WG patients, and inactive WG patients. L stands for limited WG, G for generalized WG.

Neutrophil activation and IgG subclasses of ANCA

ANCA induced neutrophil activation is dependent on Fcγ-receptors on the neutrophil, in particular FcγRII (8). Since the various IgG subclasses have different affinities for the individual Fc-receptors, we studied the relation between IgG subclass distribution in the IgG preparations, and their capacity to induce the respiratory burst. In all serum samples we measured the amount of total IgG class of ANCA and of the various IgG subclasses of ANCA, i.e. IgG1-, IgG3-, and IgG4 subclasses of ANCA, all by Elisa. Higher levels of IgG ANCA were found in sera from active disease than from inactive disease. To study relative changes in the distribution of the IgG-subclasses of ANCA irrespective of ANCA titer, we expressed the data as relative values, i.e. the quantity of ANCA of a particular subclass of IgG divided by the total amount of IgG.

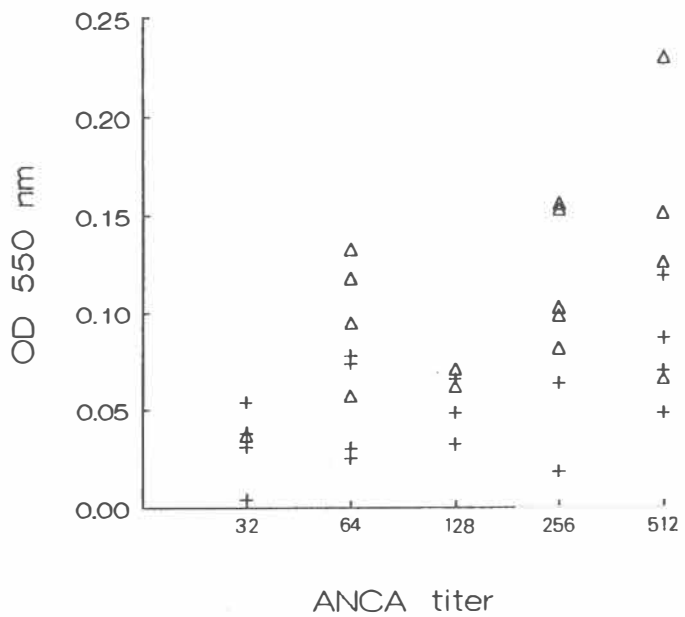

Figure 2. ΔOD 550 nm as a measure for the amount of superoxide production by normal PMNs induced by the IgG fractions in relation with the reciprocal titer of ANCA. The triangles stand for samples drawn during active disease, the plusses stand for samples drawn during remission.

In most cases, samples from active disease had a higher relative value of IgG3 subclass of ANCA than samples from inactive disease. This was not observed for IgG1- and IgG4-subclasses of ANCA. Next, we related the relative change of IgG-subclasses of ANCA from inactive to active disease, i.e. the ratio of their relative values during active and inactive disease, to the percentual increase in neutrophil activating capacity from inactive into active disease (fig 3A,B,C): The latter appeared to be correlated with the relative change of IgG3-subclass of ANCA ($r=0.851$, $p<0.001$) but not with the relative changes of IgG1- ($r=0.382$, NS) or IgG4-subclass of ANCA ($r=0.117$, NS).

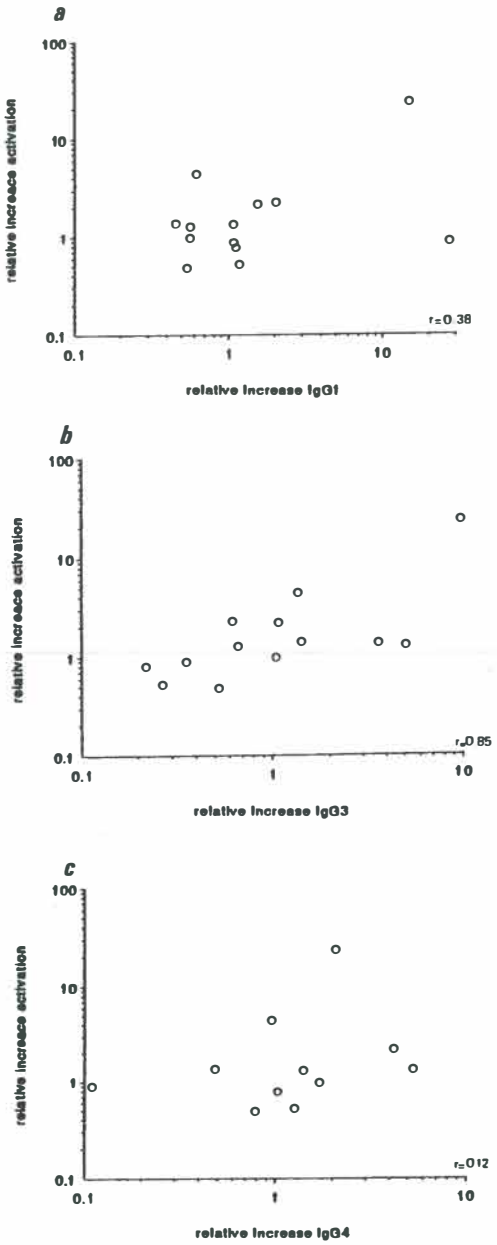

Figure 3. Percentual increase in activating capacity from inactive to active disease related to relative increase in IgG subclasses of ANCA. A. IgG1, B. IgG3, C. IgG4.

Discussion

In the present study we have demonstrated that ANCA positive IgG samples at the moment of active WG induced higher level of the respiratory burst than IgG samples from serum samples at remission. The amount of superoxide production appeared to be dependent on the amount of ANCA IgG present. In addition we observed, comparing IgG fractions with identical ANCA titers, that IgG from active disease induced the respiratory burst better, than IgG from inactive disease. The relative increase in activating capacity during the time of active disease correlated strongly with the increase in the relative amount of the IgG3-subclass of ANCA, but not with changes in the relative amounts of the IgG1 and IgG4 subclasses of ANCA.

A pathogenetic role for ANCA has been proposed by studies of Falk et al.(6) in which ANCA were demonstrated to activate neutrophils that were pretreated ("primed") with low dosage TNFα to the production of reactive oxygen species and the release of lysosomal enzymes. The activation of neutrophils by ANCA is dependent on the expression of the ANCA antigens at the cell surface of primed granulocytes. Falk's original report suggests that activation can be induced by $F(ab')_2$ fragments of ANCA. We recently demonstrated that the Fc-region of ANCA is, however, involved as well (8). Blockade of Fcγ-receptors of the neutrophil, in particular FcγRII, inhibited neutrophil activation induced by ANCA. Sera with relatively high levels of IgG3 subclass of ANCA preferentially activated neutrophils. These data are in line with those of Huizinga et al.(21) who observed that human neutrophils bind IgG3 complexes approximately three times faster than IgG1 complexes, whereas IgG2 and IgG4 complexes were not bound. Interestingly, it has been shown that renal exacerbations of WG are associated with increases of the IgG3 subclass of ANCA (19). In addition, Jayne et al. (22) demonstrated that ANCA of IgG3 subclass in patients with systemic vasculitis were relatively abundant during active disease whereas IgG2 subclass antibodies came up during remission.

To study the relation between ANCA induced neutrophil activation and IgG subclass distribution of ANCA paired plasma samples from patients with WG drawn during active and inactive disease were included. All plasma samples were ANCA positive and were able to induce the respiratory burst in primed granulocytes. A significant correlation was observed between the amount of superoxide produced and the titer of the antibodies. This confirms data in our previous study that ANCA induced neutrophil activation is dose-dependent for individual sera. However, studying superoxide production in relation to ANCA titer in the various sera, we observed that ANCA IgG derived from serum samples during active disease better induced the neutrophil respiratory burst than ANCA IgG derived from serum samples during inactive disease even when we compared sera with identical titer. As a consequence, in individual patients this relative increase in activating capacity during active disease was not related to the relative increase in ANCA.

We hypothesized, that the higher capacity of ANCA to induce the respiratory burst during active disease irrespective of titer, could be either a result of changes in the affinity of ANCA, in the epitopes recognized or in the subclasses distribution of ANCA. Since all IgG subclasses have different affinities for the Fc receptors, we favored the latter hypothesis. To correct for overall changes in antibody quantities, the percentual increase in neutrophil activating capacity from inactive to active disease was related to the relative

change of IgG subclasses of ANCA from inactive to active disease. The relative change of IgG subclasses was defined by the ratio of the relative values, i.e. the amount of ANCA of a particular IgG subclass divided by the total amount of IgG ANCA during active and inactive disease. We did not analyze IgG2 subclass of ANCA since a previous study by Brouwer et al. (19) had shown the infrequency of IgG2 ANCA in patients with WG. Comparing the paired plasma samples for each patient individually, a strong correlation was observed between the percentual increase in neutrophil activating capacity and the relative change of IgG3-subclass of ANCA, but not with the relative changes in IgG1- or IgG4-subclass of ANCA. These data strongly suggest that IgG3 ANCA facilitate granulocyte activation. Two observations subscribe the particular role of IgG3 ANCA in granulocyte activation. First, IgG3 antibody complexes bind to FcγII-receptors approximately three times faster than IgG1 antibodies, and far faster than IgG2 and IgG4 antibodies (21). Secondly, the IgG3 molecule is the most flexible molecule of the IgG family, making it conceivable that immune complex formation at the surface of the granulocyte in which IgG3 molecules are involved, facilitates neutrophil activation.

Whether or not changes in IgG3 subclass of ANCA and changes in ANCA titer are solely responsible for the fact that ANCA during active disease better induced the respiratory burst in primed neutrophils than ANCA in inactive disease cannot be deducted from this study. Different quality of the antibodies may be of importance for the pathogenesis of WG. Recently, Brouwer et al. (23) demonstrated that activated neutrophils, as assesed by their in situ H_2O_2 production, are present in renal biopsies from patients with WG. They found a strong correlation between the extent of renal impairment and the numbers of activated neutrophils producing H_2O_2, which suggests that activated PMN contribute to the pathogenesis of renal lesions in WG. However, they found no relation between the numbers of H_2O_2 producing cells present within the renal biopsy and the in vitro capacity of ANCA to activate primed granulocytes. This also suggests that other factors besides ANCA are probably important for the in vivo outcome. Dolman et al. (24) recently demonstrated that c-ANCA have an inhibitory effect on the complexation of proteinase 3 and its major physiologic inhibitor, α1-antitrypsin (α1AT), and, more importantly, that this inhibitory effect of c-ANCA correlated with clinical activity of WG but not with total c-ANCA levels.

The PMN-activating capacity of ANCA was not related to renal impairment, and occurred in patients with generalized WG as well as in patients with limited WG. In this study we demonstrate that not only ANCA titers change in the course from active to inactive disease, but that the IgG subclass distribution of ANCA changes as well. The latter changes may be of pathophysiological importance, since the relative increase in IgG3-subclass of ANCA correlated strongly with the percentual increase in activing capacity of ANCA IgG.

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CHAPTER 7

GENERAL DISCUSSION

Granulocyte-specific antinuclear antibodies (GS-ANA) have been described since 1959 (1-5). The detection of ANCA (6,7) resulted in renewed interest in those disorders in which GS-ANA had been described earlier. This thesis shows that (peri)nuclear ANCA (p-ANCA) can be detected in sera from patients with rheumatoid arthritis (RA), autoimmune liver diseases, inflammatory bowel diseases (IBD) and juvenile chronic arthritis (JCA), all diseases in which GS-ANA has been described (5). Our studies also demonstrate that most of the antibodies in these disorders are indeed directed against cytoplasmic constituents and, as such, cannot longer be regarded as GS-ANA. In JCA, however, antibodies remain that produce nuclear fluorescence on paraformaldehyde fixed neutrophils. Their presence was related with the presence of ANA, but only a minority fullfilled the definition for GS-ANA.
The antigenic specifcities of ANCA in chronic inflammatory disorders are different from those of ANCA in vasculitic disorders (8-10). In particular, the antigens recognized by ANCA in the aforementioned diseases are not proteinase 3 (PR3), myeloperoxidase (MPO) or elastase (HLE). Our studies demonstrate that lactoferrin (LF) is one of the major antigens in chronic inflammatory disorders. In addition, combinations of two polypeptides are recognized by ANCA in those disorders: a 67/66 kD doublet and a 63/54 kD doublet. These polypeptides are of unknown identity, but they seem myeloid specific. The 67/66 kD doublet probably is a granular protein since it could be demonstrated in a degranulate of neutrophils. Remarkably all of these three antigens are recognized by ANCA in the various inflammatory disorders, although the 63/54 kD doublet is recognized only sporadically in autoimmune liver diseases, Crohn's disease and JCA.

Prevalence of ANCA in the various inflammatory disorders
p-ANCA directed to antigens other than myeloperoxidase and elastase, the antigens recognized by p-ANCA in systemic vasculitides, seem of little diagnostic significance since they can be detected in a number of disorders characterized by chronic inflammation. We detected p-ANCA in 70% of rheumatoid arthritis (RA) sera, in 79% of primary sclerosing cholangitis (PSC) sera, in 88% of autoimmune chronic active hepatitis (AI-CAH) sera, in 28% of primary biliary cirrhosis (PBC) sera, in 49% of ulcerative colitis (UC) sera, in 40% of Crohn's disease (CD) sera, and in 35% of juvenile chronic arthritis (JCA) sera. The absence of p-ANCA in disease controls and normal sera suggests, however, that p-ANCA are a marker for chronic idiopathic inflammatory disorders.

ANCA and disease activity
To study if ANCA are of possible pathophysioslogical importance in the disorders studied we related the presence of ANCA with disease activity. In neither of the diseases we found a higher prevalence of ANCA in active disease compared to inactive disease. Prospective studies were, however, not performed. Nevertheless, in patients with ulcerative colitis higher titers of ANCA were observed in sera drawn during active disease than in those from inactive disease. In juvenile chronic arthritis (JCA) patients we found that ANCA are far less frequently detected in patients in remission than in patients with active disease. Thus, there is some indication that ANCA are indeed related to disease activity, at least in UC and JCA. The presence of ANCA in rheumatoid arthritis (RA) and autoimmune liver disease including primary sclerosing cholangitis (PSC), primary biliary cirrhosis (PBC) and autoimmune chronic active hepatitis (AI-CAH), was related with

disease duration or severity of disease as indicated by the presence of cirrhosis. This suggests that ANCA are rather a secondary phenomenon related to disease duration or disease progression. Another indication that ANCA in (some of these) idiopathic inflammatory disorders might be an epiphenomenon of chronic inflammation can be derived from the fact that ANCA titers in individual patients only vary to a limited extent. In PSC patients studied before and after livertransplantation levels of ANCA more than two years after removal of the diseased organ did not differ from pre-transplant values. Preliminary data suggest that this might be the same in inflammatory bowel disease patients who underwent colectomy.
However, extensive longitudinal studies, especially in UC and JCA, are needed to establish whether or not ANCA in idiopathic inflammatory diseases are related with disease activity.

Genetic factors and ANCA in idiopathic inflammatory disorders.
There is some evidence for a genetic susceptibility for ANCA. However, the various diseases in which ANCA are detected are associated with various HLA haplotypes. Generalized Wegener's granulomatosis is possibly associated with HLA B8 and DR2 (11, 12). Vasculitis is associated with HLA-DQw7. When occurring in combination with DR4 transiently positive tests for ANCA are likely, while in combination with DR2 persistent positivity for ANCA can be expected (13). Rheumatoid arthritis is associated with DR4, Dw14, and DR1 (14). Ulcerative colitis is associated with DR2 (15), while Crohn's disease has been associated with A24, B12, Bw61, DR4 and DQw3 (16). In autoimmune chronic hepatitis associations with the A1 B8 DR3 haplotype or with DR4 have been reported (17). Primary sclerosing cholangitis is associated with B8 DR3, and all patients are positive for DRw52A (18,19). PBC is not known to be associated with specific HLA haplotypes. For JCA the association with HLA phenotypes differs for the onset types. The early oligoarticular onset of JCA is associated with DR5 and DRw8 (20). Polyarticular onset JCA is associated with a subset of DQw1 (DQA1*0101) (21) and systemic onset JCA shows a weak association with DR4 (22). Since the various disorders associated with ANCA are associated with diverse HLA haplotypes, a direct association between ANCA and a single HLA haplotype is not likely.
Goldschmeding (23) described a family with four out of nine siblings being ANCA positive, one for anti-proteinase 3 and three for anti-myeloperoxidase, all sharing the same HLA haplotype: A33, B27, Cw2, DR11, DRw52, DQw3. Only the anti-proteinase 3 positive patient suffered from WG. In addition, Shanahan et al. (24) described that ANCA are more often detected in sera from family members of patients with UC than in normal control sera, indicating a proneness to produce ANCA probably linked to HLA-related inheritable or environmental factors. Whether or not this proneness extends to development of ANCA related diseases remains to be elucidated.
Another interesting observation on the expression of ANCA and genetic background was reported by Oudkerk Pool et al. (25). He and his french colleague Reumaux studied the prevalence of ANCA in european patients with inflammatory bowel disease in a centre with a high prevalence of ANCA in IBD and a centre with a low prevalence of ANCA. They concluded that differences were not due to technical disparities but that regional differences in ANCA prevalence exist in UC. This suggests that genetic and/or environmental factors are involved in the development of the antibodies.

Induction of ANCA

The induction of autoimmunity is often supposed to be related with microbial infections (26-27). Possible mechanisms involved include: molecular mimicry, stimulation of T cells by bacterial superantigens, and chronic cytokine release by infiltrating activated immunocompetent cells.

Concerning molecular mimicry bacterial heat shock proteins (HSP) are focus of interest since they are highly homologous to their human analogues. The ubiquitous distribution and high level of expression of HSP in pathogens may serve as a universal signal for the immune system to respond to infection. However, in the case of molecular mimicry this response could be direced against self antigens (28-30). The experimental evidence for involvement of HSP in autoimmune disease is mainly derived from animal models such as the adjuvant arthritis model (31), the streptococcal cell wall arthritis model (32), collagen-induced arthritis (33) and autoimmune diabetes (34) in mice. Concerning the human situation it is interesting to notice that anti-HSP antibodies are present in a number of autoimmune diseases including RA (35,36).

Recently, it has been demonstrated that a series of autoantigens, including myeloperoxidase, shows homology with the human hsp60 (37). More previously, it has been described that anti-lactoferrin antibodies bind to mycobacterial hsp65 expressed on the surface of mycobacteria, and, indeed, homology between mycobacterial hsp65 and lactoferrin could be detected (38,39) The significance of this cross-reactivity with HSP is not clear at the moment, but a possible relation between ANCA associated diseases and microbial infection should be kept in mind. Several of the idiopathic inflammatory diseases studied in this thesis have since a long time been associated with mycobacterial infections (40,41). On the other hand, Stegeman et al.(42) recently demonstrated that patients with chronic nasal carriage of Staphylococcus aureus were more prone to (ANCA-associated) relapses of WG than non-carriers, which suggests that Staphylococcal antigens either specifically or in their role as superantigens are involved in the expression of WG and/or ANCA.

Prelinary data have shown that ANCA positive JCA patients generally have high levels of anti-hsp60 antibodies, in contrast to the ANCA negative patients, although ANCA positivity and anti-hsp60 positivity were not always simultaneously present. In addition, anti-hsp antibodies are detected in juvenile diabetes mellitus as well, while we could hardly detect ANCA in this disorder. Therefore, the relation of ANCA with microbial infections and heat shock proteins deserves more attention the coming years.

Pathophysiological significance of ANCA

A pathophysiological role for ANCA has been suggested but not definetely proven. With respect to ANCA in inflammatory disorders, it will be necessary to elucidate the target antigens in order to study the possible pathophysiological role of ANCA in those disorders. For ANCA of defined specificities evidence exists that ANCA are of pathogenetic importance.

a. ANCA and function of their target antigens

Dolman et al. (43) recently demonstrated that anti-proteinase 3 antibodies exhibit an inhibitory effect on the complexation of proteinase 3 and its physiological inhibitor α_1-antitrypsin. Some of the complexes of anti-PR3 and PR3 retained elastinolytic activity.

Most anti PR3-positive sera, however, inactivated the enzyme as well, but it was speculated that the antigen-antibody complexes might dissociate at the site of inflammation resulting in enzymatic activity of PR3 at this site.
One of the ANCA antigens recognized by ANCA in idiopathic inflammatory diseases is lactoferrin. The biological role of lactoferrin in vivo is not completely resolved. It seems, however, to be bi-functional. Apo-lactoferrin, as it is degranulated by the neutrophilic granulocyte, prevents the growth of micro-organisms by depriving them from essential iron (44-46). Next, iron-saturated lactoferrin might provide iron to catalyze the Haber-Weiss reaction to produce hydroxyl radicals (47) which helps to kill the microorganisms (48). It will be interesting to study the role of anti-lactoferrin antibodies in this system. Since binding of iron by lactoferrin induces a conformational change (49) has to be investigated whether anti-lactoferrin antibodies recognize both apo- and iron saturated forms of lactoferrin, whether they prevent the iron binding and as such inhibit efficient killing.

b. ANCA and granulocyte activation
Falk et al.(50) demonstrated that ANCA can induce, in vitro, the respiratory burst in primed normal granulocytes. Expression of the ANCA antigens on the neutrophil surface is essential for this process. They postulated that binding of $F(ab')_2$ fragments of ANCA can induce the respiratory burst, an event that suggests that the ANCA antigens bind to specific or non-specific receptors on the neutrophil surface, after which $F(ab')_2$ binding results in signal transduction. Not much is known about specific receptors for the ANCA antigens. Although lactoferrin receptors or lactoferrin binding protiens have been reported for a variety of cells (51,52), the molecular structure and binding properties of these putative receptors varies considerably. In many cases the binding of this protein is relatively non-specific and may find an origin in the pI of the protein.
Our studies demonstrate that the complete ANCA IgG molecule (either anti-PR3, anti-MPO or anti-LF) is necessary for induction of the respiratory burst, indicating that in situ immune complex formation results in activation by way of Fcγ-receptors. Whether this process can take place on a single cell or aggregates of cells are needed, remains to be studied.
Curiously, not all ANCA positive samples were able to induce the respiratory burst in our assays. This was irrespective of the ANCA titer, although a relation between the amount of superoxide produced and ANCA titer was generally present. Further studies demonstrated that IgG3 subclass of ANCA may play a crucial role in the activation process. Interestingly, Stegeman et al. (53) demonstrated that the persistent presence or increase of the IgG3 subclass of ANCA in patients with WG can be used as a marker for patients with relapsing disease, again an indication that the IgG3 subclass of ANCA plays an important role in disease activity. IgG subclass studies have to be performed in the ANCA associated diseases to elucidate whether or not the incapability of some ANCA positive sera to induce the respiratory burst can explained solely by the distribution of the IgG subclasses of ANCA. Few studies have been published on this subject so far. Reumaux et al. (54) reported that ANCA in IBD are predominantly of the IgG1 and IgG3 subclasses, but did not evaluate the subclass distribution in relation to antigenic specificities of ANCA or their granulocyte activating capacities.
Whether these in vitro studies can be translated to the in vivo situation remains to be

proven. Some data, however, provide indirect evidence. Csernok et al. (55) reported the presence of primed neutrophils in the peripheral blood of patients with active Wegener's granulomatosis. Pro-inflammatory cytokines, as priming agents, have been reported to occur in the sera of patients with WG, RA, IBD, and autoimmune liver diseases (56-60). In addition, Brouwer et al. (61) demonstrated the presence of activated neutrophils in renal biopsies from patients with Wegener's granulomatosis. The strong correlation they observed between the extent of renal impairment and the numbers of activated neutrophils producing H_2O_2 suggests that activated PMN contribute to the pathogenesis of renal lesions in WG.

Concluding remarks

In conclusion, we suggest that the granulocyte-specific antinuclear antibodies (GS-ANA) in rheumatoid arthritis, autoimmune liver diseases, and inflammatory bowel diseases are, indeed, anti-neutrophil cytoplasmic antibodies. In juvenile chronic arthritis ANCA do occur, but the presence of real GS-ANA cannot be excluded. The prevalence of ANCA in the inflammatory disorders mentioned before seems of restricted diagnostic significance. With respect to the inflammatory bowel diseases, the presence of ANCA, in particular in high titer, appears to be related with active ulcerative colitis.

The autoantibodies in these idiopathic inflammatory disorders do not recognize previously known ANCA antigens in systemic vasculitis such as proteinase 3 and myeloperoxidase. Besides lactoferrin, combinations of uncharacterized neutrophil granule constituents are targets for ANCA in the inflammatory diseases mentioned before. The presence of ANCA, although not studied prospectively, does not show a clear relation with disease activity in these disorders, possibly with the exception of ulcerative colitis. ANCA appears to be related to the chronicity of the diseases, suggesting that these autoantibodies are possibly the result of chronic inflammation. A pathophysiological role for the autoantibodies is suggested by the fact that they are able to activate primed neutrophils. This could mean that, although the antibodies are possibly the result of chronic inflammation and are not causative for the disease in any way, their presence results in more severe or more chronic inflammation due to their capacity to activate and degranulate neutrophils. This results in abundance of the antigen, and conceivably in antibody production which again leads to neutrophil activation: the vicious circle of chronic inflammation?

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SUMMARY

This thesis studies the prevalence of anti-neutrophil cytoplasmic antibodies (ANCA) in idiopathic inflammatory disorders in which GS-ANA were described during the 60s: rheumatoid arthritis, autoimmune liver diseases, inflammatory bowel disease, and juvenile chronic arthritis. In all of these disorders the presence of ANCA was related to disease activity, disease duration and severity of diseae. Furthermore, the antigenic specificity of ANCA in chronic inflammatory disorders was studied. In addition, this thesis investigated the pathophysiological significance of ANCA in chronic inflammatory disorders, by studying the capacity of ANCA to activate granulocytes.

Chapter 1 is a review in which target antigens of ANCA and the clinical significance of the different ANCA specificities are discussed. The classical or cytoplasmic ANCA (c-ANCA) is in almost all of the cases directed against proteinase 3. C-ANCA occur in more than 90% of patients with extended Wegener's granulomatosis (WG), in 75% of patients with limited WG, and in some 40 to 50% of patients with vasculitic overlap syndromes. The presence of c-ANCA is highly specific for those diseases and changes in levels of c-ANCA precede disease activity. Perinuclear ANCA (p-ANCA) are directed against different cytoplasmic constituents of neutrophils and occur in a wide range of diseases. p-ANCA directed against myeloperoxidase have a high specificity for necrotizing vasculitides, but may incidentally occur in other diseases as well. p-ANCA directed against elastase are incidentally found in patients with vasculitic disorders, whereas lactoferrin antibodies are detected in patients with primary sclerosing cholangitis with or without ulcerative colitis, and in rheumatoid arthritis. p-ANCA of undefined specificity can be detected in ulcerative colitis, Crohn's disease, autoimmune liver diseases, chronic arthritides and in some 5% of healthy controls. The diagnostic value of p-ANCA of undefined specificity has to await characterization of the antigens involved.

Chapter 2 studies the prevalence, interrelationships and target antigens of ANCA in rheumatoid arthritis, and relates the presence of ANCA to disease duration and occurrence of extra-articular manifestations. ANCA were detected in 70% of rheumatoid sera, wheras in 36% of the sera the antibodies reacted with cytoplasmic constituents of the neutrophil as shown by a cytoplasmic staining pattern on paraformaldehyde fixed granulocytes. Elisa studies showed that 20% of the sera reacted with lactoferrin, 1% with myeloperoxidase, and 1% with elastase. Western blotting studies confirmed the Elisa data and revealed reactivity with hitherto unknown polypeptides of 67/66 kD (6%) and 63/54 kD (9%). Neither of the antibodies was associated with a particular subset of disease, but the prevalence of the antibodies tended to increase with longstanding disease. Anti-lactoferrin antibodies were demonstrated to be present in synovial fluid as well.

Chapter 3.1 studies the diagnostic significance of ANCA in chronic liver diseases by testing sera from patients with primary biliary cirrhosis (PBC), primary sclerosing cholangitis (PSC), autoimmune chronic hepatitis (AI-CAH) and non-autoimmune liver diseases. ANCA were detected in 28% of PBC sera, in 79% of PSC sera, and in 88% of AI-CAH sera, while they were not detected in non-autoimmune liver disease sera. The presence of ANCA was significantly correlated with the presence of cirrhosis. The ANCA antigens involved were not Pr3, MPO or HLE, but Western blotting studies showed reactivity with lactoferrin, the 67/66 kD doublet and a 40 kD protein. Reactivity with either of these proteins was observed in sera from patients with PBC (20%), PSC (38%) and AI-CAH (17%).

Chapter 3.2 approaches the possible immunopathogenetic importance of ANCA in PSC by

studying the occurrence of ANCA in PSC before and after liver transplantation. Nine patients with PSC, all positive for p-ANCA, showed a decline in ANCA titer immediately after liver transplantation, but during follow-up ANCA titers equal to pre-transplantation titers were measured. No recurrence of disease could be observed, as judged by liver histology. Control patients were ANCA negative, and remained ANCA negative after liver transplantation. This study demonstrates .hat ANCA remain present after liver transplantation and that the synthesis of ANCA is not related to the presence of the diseased organ.

Chapter 4.1 studies the prevalence of ANCA in inflammatory bowel diseases (IBD). p-ANCA can be demonstrated in 49% of patients with ulcerative colitis (UC), and in 40% of patients with Crohn's disease (CD). Titers of ANCA are higher in patients with UC compared to CD patients. The antigenic specificity of ANCA in IBDas tested by Elisa is generally unknown, although incidental sera recognize lactoferrin or myeloperoxidase. So, within the spectrum of IBD, the presence of p-ANCA is not specific for UC. When titers of ANCA are taken into account, the presence of high-titered p-ANCA suggests active UC.

Chapter 4.2 studies the antigenic specificity of ANCA in IBD. 76% of the p-ANCA positive sera in UC and 50% of the p-ANCA positive sera in CD showed cytoplasmic fluorescence on paraformaldehyde fixed neutrophils, indicating that indeed cytoplasmic antigens are recognized by a considerable number of these sera. Western blot analysis showed reactivity with either lactoferrin, the 67/66 kD doublet or the 63/54 kD doublet in 46% of the UC sera and in 32% of the CD sera. Since identical patterns of reactivity have been observed in rheumatoid arthritis sera and autoimmune liver disease sera these data suggest that ANCA of restricted specificities are not specific for IBD but are present in diverse conditions characterized by chronic inflammation.

Chapter 5 describes the prevalence of ANCA in juvenile chronic arthritis (JCA) and other juvenile inflammatory disorders such as cystic fibrosis, juvenile diabetes and connective tisue diseases. p-ANCA are detected in 35% of JCA sera and in only 6% of the disease control sera. Considering the onset type of JCA, ANCA are detected in 44% of oligoarticular onset JCA, in 36% of polyarticular onset JCA, and in 16% of systemic onset JCA. No relation was observed between ANCA and either the presence of rheumatoid factor, prolonged disease or more progressive disease. However, ANCA were significantly less frequently detected during remission of JCA suggesting that ANCA might be a marker of disease activity. On paraformaldehyde fixed neutrophils only 14% of the JCA sera showed cytoplasmic fluorescence, while 23% of the sera showed nuclear fluorescence. The nuclear fluorescence pattern was related with the presence of ANA, especially in the sera of patients with polyarticular onset JCA. Antigen specificity studies showed that PR3, MPO or HLE are not the antigens involved. Western blotting studies showed reactivity with lactoferrin (5%) or polypeptides of 67/66 kD (9%).

Chapter 6.1 studies neutrophil activation by ANCA, and analyzes the underlying mechanism. Primed normal donor neutrophils are demonstrated to express the ANCA antigens PR3, MPO and lactoferrin on the cell surface, and interaction of these antigens with their respective antibodies resulted in activation of the neutrophils as demonstrated by the production of superoxide. In contrast to other reports, activation of neutrophils by ANCA depended on the presence of the Fc-part of the antibodies, and the availability of the Fcγ-receptors on the neutrophils. Especially the second Fcγ-receptor appeared to be involved. Not all ANCA positive samples were capable of granulocyte activation irrespective of the

ANCA titer. Interestingly, samples capable of granulocyte activation contained relatively high levels of the IgG3 subclass of ANCA in contrary to the ANCA positive samples that could not induce activation.

Chapter 6.2 extends the studies of chapter 6.1. Paired ANCA positive samples from active and inactive disease were studied for their capacity to induce the respiratory burst, and granulocyte activation was related to ANCA titer and subclass distribution, together with disease activity. Sera of patients with active disease better induced the respiratory burst in primed neutrophils than sera of patients with inactive disease. There was a significant relation between ANCA titer and the amount of superoxide produced. In addition, changes in IgG3-subclass of ANCA appeared to correlate with changes in superoxide production. Therefore, not only changes in ANCA titer during the course of disease, but also changes in levels of IgG3-subclass of ANCA are important as the latter are directly related to the neutrophil activating capacity of the autoantibodies.

The final chapter of this thesis, ***chapter 7***, is an overall discussion on the data generated in this thesis. It concludes with the vicious circle of ANCA associated idiopathic inflammatory disorders: ANCA are capable of activation of primed neutrophils. This results in respiratory burst and degranulation. The ANCA antigens are now availalbe for immune complex formation and new antibodies can be produced.

With respect to the questions posed in ***chapter 1.2*** we conclude:

1. ANCA in the idiopathic inflammatory disorders have comparable antigenic specificities, in particular lactoferrin and polypeptide doublets of 67/66 and 63/54 kD, suggesting that these ANCA are not specific for one disease entity. ANCA of those specificities rather seem a marker for inflammatory disorders on an autoimmune background.
2. ANCA in rheumatoid arthritis and autoimmune liver diseases are related to either disease duration or severity of disease. This relation is less clear for ANCA in the inflammatory bowel diseases and juvenile chronic active hepatitis.
3. The pathogenetic role of ANCA remains in part to be elucidated. Nevertheless, ANCA can activate primed neutrophils. This process is Fc-dependent and particularly exerted by the IgG3 subclass of ANCA. These data suggest that ANCA are capable of maintaining or even amplifying the inflammatory process.

SAMENVATTING

Dit proefschrift omvat een studie naar de prevalentie van anti-neutrofiel cytoplasmatische antistoffen (ANCA) in idiopathische ontstekingsziekten waarbij, gedurende de zestiger jaren, granulocyt-specifieke anti-nucleaire antistoffen (GS-ANA) zijn beschreven: reumatoide artritis, autoimmuun lever aandoeningen, inflammatoire darm aandoeningen en juveniele chronische artritis. De aanwezigheid van ANCA werd gerelateerd aan activiteit van de ziekte, duur van de ziekte en ernst van de ziekte. Tevens werd de antigene specificiteit van ANCA in chronische ontstekingsziekten bestudeerd. Ook werd onderzoek gedaan naar de pathofysiologische betekenis van ANCA, met name naar het vermogen van de autoantistoffen om granulocyten te activeren.

Hoofdstuk 1 is een overzicht waarin de doelwit antigenen van ANCA en de klinische relevantie van de verschillende ANCA specificiteiten wordt besproken. De klassieke of cytoplasmatische ANCA (c-ANCA) is bijna altijd gericht tegen proteinase 3. c-ANCA zijn aanwezig in meer dan 90% van de patienten met gegeneraliseerde ziekte van Wegener, in 75% van de patienten met gelimiteerde ziekte van Wegener, en in ongeveer 40 tot 50% van de patienten met microscopische polyangiitis. c-ANCA is in hoge mate specifiek voor deze aandoeningen, en veranderingen in c-ANCA titers gaan vooraf aan periodes van ziekte activiteit. De perinucleaire ANCA (p-ANCA) is gericht tegen diverse cytoplasmatische bestanddelen van de neutrofiel, en wordt bij verschillende aandoeningen gevonden. p-ANCA gericht tegen myeloperoxidase hebben een hoge specificiteit voor necrotizerende systemische vasculitis, maar kunnen incidenteel bij andere aandoeningen voorkomen. p-ANCA gericht tegen elastase worden incidenteel gedetecteerd in patienten met systemische vasculitis, terwijl lactoferrine antistoffen gevonden worden bij patienten met primair scleroserende cholangitis, al dan niet in combinatie met colitis ulcerosa, en in reumatoide artritis. p-ANCA van onbekende specificiteit wordt onder andere gevonden bij patienten met colitis ulcerosa, de ziekte van Crohn, autoimmuun lever aandoeningen, chronische artritiden en bij ongeveer 5% van de gezonde bevolking. De diagnostische betekenis van p-ANCA van onbekende specificiteit wacht op karakterisatie van de betrokken antigenen.

Hoofdstuk 2 bestudeert de prevalentie en de betrokken antigenen van ANCA in reumatoide artritis, en correleert de aanwezigheid van ANCA met ziekteduur en met het voorkomen van extra-articulaire manifestaties. ANCA werden gedetecteerd in 70% van de sera bij reumatoide artritis. Bij 36% van de sera kon daadwerkelijk worden bewezen dat deze ANCA gericht waren tegen een cytoplasmatisch bestanddeel van de granulocyt. Elisa studies toonden dat 20% van de sera antistoffen bevatten gericht tegen lactoferrine, 1% tegen myeloperoxidase en 1% tegen elastase. Western blotting studies bevestigden de Elisa resultaten, en toonden verder reactiviteit van de sera met tot nog toe onbekende polypeptiden van 67/66 kD (6%) en 63/54 kD (9%). Geen van de antistoffen was geassocieerd met een specifieke subset van de ziekte, maar het voorkomen van de antistoffen leek gerelateerd met ziekteduur. ANCA gericht tegen lactoferrine werden tevens aangetoond in de synoviale vloeistof.

Hoofdstuk 3.1 is een studie naar de diagnostische betekenis van ANCA bij chronische lever-aandoeningen. Sera van patienten met primaire biliaire cirrose (PBC), primair scleroserende cholangitis (PSC) en autoimmuun chronisch actieve hepatitis (AI-CAH) werden bestudeerd, in combinatie met sera van niet-autoimmuun lever-aandoeningen. ANCA werden gedetecteerd in 28% van de PBC sera, in 79% van de PSC sera en in 88% van de AI-CAH sera, terwijl ze niet werden gevonden in de sera van niet-autoimmuun

leveraandoeningen. De aanwezigheid van ANCA was significant gecorreleerd met de aanwezigheid van cirrose. De betrokken ANCA antigenen waren niet proteinase 3, myeloperoxidase of elastase, maar Western blotting studies toonden reactiviteit van de ANCA met lactoferrine, het 67/66 kD doublet en een 40 kD eiwit. 20% van de PBC sera, 38% van de PSC sera en 17% van de AI-CAH sera toonden reactiviteit met een van deze eiwitten.

Hoofdstuk 3.2 beschrijft een studie naar de mogelijke immuno-pathogenetische betekenis van ANCA in PSC door het voorkomen van ANCA in PSC zowel vóór als na lever transplantatie te bestuderen. Negen patienten met PSC, allen positief voor ANCA, toonden onmiddelijk na lever transplantatie een daling in hun ANCA titer, maar gedurende het vervolg werden deze titers weer gelijk aan die van vóór transplantatie. Terugkeer van PSC, beoordeeld aan de hand van lever histologie, werd niet waargenomen. Controle patienten waren negatief, en bleven negatief voor ANCA na levertransplantatie. Deze studie toont dat ANCA nog aanwezig zijn na lever transplantatie, en dat de synthese van ANCA niet gerelateerd is aan de aanwezigheid van het zieke orgaan.

Hoofdstuk 4.1 bestudeert de prevalentie van ANCA bij inflammatoire darm-aandoeningen. ANCA zijn aanwezig bij 49% van de patienten met colitis ulcerosa (UC), en bij 40% van de patienten met de ziekte van Crohn (CD). ANCA titers waren hoger in patienten met UC vergeleken met CD. De antigene specificiteit van ANCA bij deze darm-aandoeningen is grotendeels onbekend, hoewel incidentele sera reageren met lactoferrine of myeloperoxidase. Wanneer de ANCA titers in aanmerking worden genomen, dan suggereert de aanwezigheid van een hoge titer van ANCA de diagnose "actieve UC".

Hoofdstuk 4.2 is een studie naar de antigene specificiteit van ANCA bij inflammatoire darm-aandoeningen. 76% van de p-ANCA positieve sera bij UC, en 50% van de p-ANCA positieve sera bij CD vertoonden cytoplasmatische fluorescentie op paraformaldehyde gefixeerde granulocyten, hetgeen aangeeft dat een aanzienlijk aantal van deze sera inderdaad cytoplasmatische antigenen herkent. Western blotting studies toonden reactiviteit met lactoferrine, het 67/66 kD doublet of het 63/54 kD doublet bij 46% van de UC sera en bij 32% van de CD sera. Identieke reactiepatronen zijn waargenomen in sera van patienten met reumatoide artritis of autoimmuun lever-aandoeningen, hetgeen suggereert dat de aanwezigheid van ANCA met deze specificiteit niet specifiek is voor inflammatoire darm aandoeningen.

Hoofdstuk 5 is een studie naar de prevalentie van ANCA bij juveniele chronische artritis (JCA) en andere (juveniele) ontstekingsziekten zoals cystic fibrosis, juveniele diabetes en juveniele bindweefselziektes. p-ANCA werden aangetroffen in 35% van de JCA sera, en slechts in 6% van de ziekte-controle sera. Wanneer het onset type van JCA in ogenschouw wordt genomen, dan zijn ANCA in 44% van de sera van patienten met oligoarticulaire begin van JCA aanwezig, in 36% van de sera van patienten met polyarticulair begin van JCA en in 16% van de sera van patienten met een systemisch begin van de ziekte. De aanwezigheid van ANCA was niet gecorreleerd met de aanwezigheid van reumafactor, verlengde ziekteduur of ernstiger ziekte. Echter, ANCA werden significant minder waargenomen tijdens remissie van de ziekte. Het zou dus mogelijk kunnen zijn dat ANCA een merker voor ziekte-activiteit zijn. Op paraformaldehyde gefixeerde granulocyten toonden slechts 14% van de JCA sera cytoplasmatische fluorescentie, terwijl 23% van de sera een kern aankleuring gaven. Deze kernaankleuring was gecorreleerd met de aanwezigheid van anti-nucleaire antistoffen. Studies naar de antigene specificiteiten van

ANCA toonden dat proteinase 3, myeloperoxidase of elastase niet de betrokken antigenen zijn. Western blotting studies toonden reactiviteit met lactoferrine (5%) en polypeptides van 67/66 kD (9%).

Hoofdstuk 6.1 is een studie naar neutrofiel activatie door ANCA en naar het onderliggende mechanisme. Aangetoond werd dat "geprime-de" granulocyten de ANCA antigenen PR3, MPO en lactoferrine op hun celoppervlak tot expressie brengen, en dat interactie van deze antigenen met de respectieve antilichamen resulteert in activatie van de granulocyt (gemeten aan de hand van de superoxide produktie). In tegenstelling tot andere studies vonden wij dat activatie van granulocyten door ANCA afhankelijk is van de aanwezigheid van de Fc-staart van het antilichaam en de expressie van Fc-receptoren op het oppervlak van de neutrofiel. Met name de Fcγ-RII lijkt betrokken bij dit proces. Niet alle ANCA positieve sera waren in staat tot neutrofiel activatie, ongeacht de ANCA titer. Echter, sera die in staat waren tot granulocyten activatie bevatten relatief hoge IgG3-ANCA spiegels in tegenstelling tot sera die geen activatie gaven.

Hoofdstuk 6.2 is een uitbreiding van hoofdstuk 6.1. Nu werden gepaarde ANCA monsters van actieve en inactieve fases van de ziekte bestudeerd naar hun vermogen om granulocyten te activeren. De mate van activatie was gecorreleerd met de ANCA titer, hoewel ANCA monsters uit de actieve fase van de ziekte een grotere activatie van granulocyten te weeg brachten dan ANCA monsters met een zelfde titer uit een niet-actieve fase. Veranderingen in het vermogen van ANCA tot inductie van superoxide produktie door granulocyten waren binnen de individuele patient sterk gecorreleerd met veranderingen in de spiegels van IgG3 ANCA.

Hoofdstuk 7 stelt diverse onderdelen van dit proefschrift ter discussie en besluit met een beschrijving van de vicieuze cirkel welke voorkomt bij ANCA geassocieerde idiopathische ontstekingsziekten. ANCA zijn in staat om "geprime-de" granulocyten aan te zetten tot zuurstofradicaal productie en degranulatie. Hierbij komen de ANCA antigenen beschikbaar voor immuuncomplex vorming en nieuwe antistof vorming. Mogelijk blijft op deze manier de ontstekingsreactie in stand gehouden.

Als antwoord op de vragen gesteld in hoofstuk 1.2 kan geconcludeerd worden:

1. ANCA in de verschillende ontstekingsziekten hebben vergelijkbare antigene specificiteiten, zoals lactoferrine en de 67/66 en 63/54 kD polypeptide doubletten, en dus zijn deze ANCA niet specifiek voor één aandoening. Het lijkt erop alsof ze een merker zijn voor ontstekingsziekten met een auto-immuun achtergrond.
2. Uit deze studies kan verder worden geconcludeerd dat ANCA bij reumatoide artritis en autoimmuun-leveraandoeningen gerelateerd zijn met ziekte-duur of -ernst. Voor ANCA bij autoimmuun-darmaandoeningen en juveniele chronische artritis is deze relatie minder duidelijk.
3. De pathogenetische rol voor ANCA moet nog verder worden opgehelderd. Echter, ANCA kunnen granulocyten activeren. Dit proces is Fc-afhankelijk en met name de IgG3 subklasse van ANCA speelt hier een belangrijke rol in. Deze data suggereren dat ANCA in staat zijn de ontstekingsreactie te onderhouden of zelfs te versterken.